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(54) Title: METHODS FOR THE DIFFERENTIATION OF HUMAN STEM CELLS

(57) Abstract: Methods for differentiating human embryonic stem cells into specific cell types are disclosed herein. These methods can be used to generate synaptically active dopaminergic neurons. In one embodiment, the differentiated cells are neuronal cells that can be used in the treatment of neurodegenerative disorders such as Parkinson's disease. Methods are also disclosed herein for differentiating human embryonic stem cells into cells of the endoderm, mesoderm, and ectoderm.

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METHODS FOR THE DIFFERENTIATION OF HUMAN STEM CELLS

FIELD

This invention relates to the field of methods for the *in vitro* production of differentiated cells from stem cells, such as to the production of hepatocytes and neurons.

BACKGROUND

Neurons in the central and peripheral nervous systems degenerate as a normal function of human development and aging. Pathological neuron degeneration, however, is a serious condition seen in several neurological disorders. Neuronal degeneration can be specific or diffuse, and can lead to sensory, motor and cognitive impairments. Neurodegenerative disorders encompass a range of seriously debilitating conditions including Parkinson's disease, amyotrophic lateral sclerosis (ALS, "Lou Gehrig's disease"), multiple sclerosis, Huntington's disease, Alzheimer's disease, Pantothenate kinase associated neurodegeneration (PKAN, formerly Hallervorden-Spatz syndrome), multiple system atrophy, diabetic retinopathy, multi-infarct dementia, macular degeneration, and the like. These conditions are characterized by a gradual but relentless worsening of the patient's condition over time. These disorders affect a large population of humans, especially older adults. Nevertheless, the understanding of these disorders is extremely limited and incomplete.

Many advances have been made in years past in gaining a better understanding of Parkinson's disease, Alzheimer's disease and Huntington's disease. The primary cause of cognitive dysfunction for all three disorders has been directly linked to neuron degeneration, usually in specific areas of the brain. Parkinson's disease is linked to degeneration of neurons in the substantia nigra, while Alzheimer's disease is in some part due to loss of pyramidal neurons in the limbic cortex (Braak, E. & Braak, H., 1999, In: V.E. Koliatsos & R.R. Ratan (eds.), *Cell Death and Diseases of the Nervous System*, Totowa, NJ: Humana Press, pp. 497-508). Huntington's disease's cognitive deficits are produced by degeneration of cells in the caudate nucleus of the striatum. However, although the symptoms and

progression of these diseases are well characterized, the causes and triggers at onset are not well understood.

Thus, several strategies are being pursued to develop new therapies for neurodegenerative disorders, including Parkinson's disease. For Parkinson's disease, the techniques range from the use of dopaminotrophic factors (Takayama et al., *Nature Med.* 1:53-58, 1995) and viral vectors (Choi-Lundberg et al., *Science* 275:838-841, 1997) to the transplantation of primary xenogeneic tissue (Deacon et al., *Nature Med.* 3:350-353, 1997). Transplantation of dopaminergic neurons is a clinically promising experimental treatment in late stage Parkinson's disease. More than 200 patients have been transplanted worldwide (Olanow et al., *Trends Neurosci.* 19:102-109, 1996), and clinical improvement has been confirmed (Olanow et al., *supra*, and Wenning et al., *Ann. Neurol.* 42:95-107, 1997) and was correlated to good graft survival and innervation of the host striatum (Kordower et al., *N. Engl. J. Med.* 332:1118-1124, 1995). However, fetal nigral transplantation therapy generally requires human fetal tissue from at least 3-5 embryos to obtain a clinically reliable improvement in the patient. A different source of these neurons is clearly needed.

Dopaminergic neurons have been generated from murine CNS precursor cells (PCT Application No. PCT/US99/16825; and Studer et al., *Nature Neurosci.* 1:290-295, 1998). These precursor-derived neurons are functional *in vitro* and *in vivo* and restore behavioral deficits in a rat model of Parkinson's disease. Even though the primary mesencephalic CNS stem cell culture can provide differentiated dopaminergic neurons suitable for use in cell therapy, the cell number provided by this method is limited. The percentage of differentiated dopaminergic neurons obtained from expanded mesencephalic precursors decreases as the cells are expanded more than about 10-100 fold. Mesencephalic precursors can generate only about 10% to 15% dopaminergic neurons (out of total cell number) after 10-100 fold expansion, and when the precursors are expanded 1000 fold, that number drops further, to only about 1%. Thus, a need clearly remains for alternate sources of these cells. In addition, there is a need for reliable methods for generating larger numbers of primate neurons.

SUMMARY

Methods for differentiating human embryonic stem cells are disclosed herein. These methods can be used to generate neurons, including, but not limited to, synaptically active dopaminergic neurons. In one embodiment, the differentiated cells can be used in the treatment of neurodegenerative disorders such as Parkinson's disease. These methods are also of use in generating endodermal cells, such as hepatocytes.

In one embodiment, a method is disclosed for culturing human embryonic stem cells to produce a population of cells comprising human neuronal cells. The method includes (1) expanding undifferentiated embryonic stem cells in the presence of fibroblast growth factor (FGF)-2 and dissociating the undifferentiated embryonic stem cells to form a population comprising a majority of single cells; (2) generating embryoid bodies from the population comprising the majority of single cells in the presence of FGF-4 and in the absence of feeder cells; (3) culturing the embryoid bodies in the presence of FGF-4 on an extra-cellular matrix to select for central nervous system precursor cells; (4) expanding the central nervous system precursor cells by culturing the central nervous system precursor cells in an expansion medium that comprises at least one of FGF-2, sonic hedgehog factor (Shh) and FGF-8, wherein the central nervous system precursor cells are not cultured on an extra-cellular matrix; and (5) differentiating the expanded central nervous system precursor cells to form a culture of differentiated neuronal cells by culturing the expanded central nervous system precursors in a culture medium that lacks FGF-4, FGF-2, Shh, and FGF-8; thereby producing the population of cells comprising at least 30% neuronal cells. In one specific example, the extra-cellular matrix includes, or consists essentially of, fibronectin. Optionally, the method also includes contacting the cells with other cells of the nervous system, such as astrocytes or glial cells. These cells can be from the same species, or from a different species (xenogeneic).

In another embodiment, a method is disclosed for culturing human embryonic stem cells to produce a population of cells comprising human neuronal cells. The method includes (1) expanding undifferentiated embryonic stem cells in

the presence of fibroblast growth factor (FGF)-2 and dissociating the undifferentiated embryonic stem cells to form a population comprising a majority of single cells; (2) generating embryoid bodies from the population comprising the majority of single cells in the presence of retinoic acid and in the absence of feeder cells; (3) culturing the embryoid bodies in the presence of retinoic acid on an extra-cellular matrix to select for central nervous system precursor cells; (4) expanding the central nervous system precursor cells by culturing the central nervous system precursor cells in an expansion medium that comprises at least one of FGF-2, sonic hedgehog factor (Shh) and FGF-8, wherein the central nervous system precursor cells are not cultured on fibronectin; (5) differentiating the expanded central nervous system precursor cells to form a culture of differentiated neuronal cells by culturing the expanded central nervous system precursors in a culture medium that lacks FGF-4, FGF-2, Shh, and FGF-8, thereby producing the population of cells comprising at least 30% neuronal cells. Optionally, the method also includes contacting the cells with other cells of the nervous system, such as astrocytes or glial cells. These cells can be from the same species as the embryonic stem cells, or from a different species (xenogeneic). In one example, the extra-cellular matrix is fibronectin.

In a further embodiment, a simplified method is disclosed for culturing human embryonic stem cells to produce a population of cells comprising human neuronal cells. The method includes (1) expanding human embryonic stem cells on a substrate coated with an extra-cellular matrix in fibroblast-conditioned medium in the absence of LIF, serum, or a serum replacement; (2) culturing the cells in medium including one or more of insulin, transferrin and selenium, in the absence of serum, for at least three days; and (3) culturing the cells in N2 medium in the absence of serum for at least three days. In one example, the extra-cellular matrix comprises, or consists essentially of laminin.

Also disclosed herein are culture conditions to differential embryonic stem cells into cells of the three embryonic germ-layers, germ cells and extra-embryonic cells.

The foregoing and other features and advantages will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1A-D are a set of digital images showing that endodermal cells expressing genes characteristic of liver migrate from EBs treated with LiCl. Fig. 1A is a digital image showing OV6 positive cells surrounding an EB. Fig. 1B is a digital image showing double positive cells for cytokeratin8 and GATA4 are abundant between two EBs. After 6 days of differentiation in Stage 3, Albumin positive cells are abundant (Fig. 1C). These albumin positive cells also express a-fetoprotein (Fig. 1C, inset). At this later time, many cells expressing the liver specific keratin, CK18, and albumin are polyploid (Fig. 1D).

Figs. 2A-D are a set of digital images showing that endodermal cells expressing genes characteristic of liver migrate from EBs treated with LiCl. Fig. 2A is a digital image showing OV6 positive cells surrounding an EB. Fig. 2B is a digital image showing double positive cells for cytokeratin8 and GATA4 are abundant between two EBs. After 6 days of differentiation in Stage 3, Albumin positive cells are abundant (Fig. 2C). These albumin positive cells also express a-fetoprotein (Fig. 2C, inset). At this later time, many cells expressing the liver specific keratin, CK18, and albumin are polyploid (Fig. 2D).

Figs. 3A-I are a protocol outline and a set of digital images showing the morphology of human ES cells during differentiation into dopaminergic neurons using the 5-stage protocol. **Fig. 3A** is a digital image of stage 1, undifferentiated hES colonies on MEF feeder cells. **Fig. 3B** is a digital image of Stage 2, simple embryoid bodies (EBs) from hES cells after 4 and 8 days (inset). After 8 days, the EBs develop an outer layer of primitive endoderm. **Figs. 3C** and **3D** are digital images of stage 3, cells migrating out of EBs (FGF4 treated EBs compared to untreated control EBs) after re-plating on adherent cell culture plates. Rosette-

forming neural precursors (**Fig. 3D**, inset, higher magnification) can only be observed after FGF4 treatment. **Fig. 3E** is a digital image of tage 4, neural precursors (FGF4 in stage 2 and 3) 8 days after re-plating on poly-L-ornithine coated plates in the presence of 20 ng/ml FGF2, 100 ng/ml FGF8 and 500 ng/ml Shh. **Fig. 3F** is a digital image of Neural precursors stained positive for nestin. **Fig. 3G** is a digital image of neuronal morphology after 8 days of differentiation in stage 5. **Fig. 3H** is a digital image of abundant expression of Tuj1 in these neurons. **Fig. 3I** is a general outline of the differentiation protocol.

Figs. 4A-Q are digital images and graphs showing the differentiation of human ES cells to neurons. **Figs. 4A-B** are digital images showing that RA treatment increases Sox1 (bright) and HB9 (dull) coexpressing motor neuron precursors in EBs and **4B**, the number of Tuj1 (bright) and HB9 (dull) coexpressing motor neurons after terminal differentiation (stage 5). **Figs. 4C-E** are digital images showing that FGF-4 treatment in stage 2 and 3 induces expression of markers for ventral midbrain precursors and dopaminergic neurons. **Fig. 4C** shows FGF4 extinguishes HB9 (dull) expression in Sox1 (bright) positive neural precursors in EBs (compare to **Fig. 4A**). **Fig. 4C** is a digital image showing the coexpression of nestin (bright) and engrailed-1 (dull) in stage 3. **Fig. 4E** is a digital image ofTuj1 (bright) and TH (dull) coexpressing dopaminergic neurons after terminal differentiation (stage 5, day 8). **Fig. 4F** is a digital image showing Lmx1b (dull) is expressed in the majority of TH (bright) positive neurons after terminal differentiation (stage 5, day 8). **Fig. 4G** is a digital image showing the proportion of Tuj1 expressing neurons after terminal differentiation (stage 5) in the presence of indicated growth factors in stage 2 and 3. **Fig. 4H** is a digital image showing that treatment of engrailed-1 positive neural precursors (FGF-4 in stage 2 and 3) with Shh and FGF8 in stage 4 significantly increases proportion of TH/Tuj1 coexpressing dopaminergic neurons after terminal differentiation (compared to FGF4-only treated control cultures). **Figs. 4I-Q** are digital images showing the effect of transplantation of human ES cell derived dopaminergic neurons in 6-OHDA lesioned rats. **Figs. 4I, 4L, 4M, 4N** are digital images demonstrating that grafts show many Tuj1 (dull) positive neurons 5 to 95 days after transplantation. Transplanted human cells were

identified by anti human nuclei antibody (bright) or human specific 70 kDA neurofilament (bright). **Figs. 4J** and **4K** are digital images showing clusters of TH expressing cells (dull) 5 days after transplantation. TH positive cells could be identified as transplanted cells by anti human nuclei (bright) coexpression. **Fig. 4N, 4O, 4P, 4Q** are digital images showing ES cell derived transplanted cells express additional neuronal astrocytic markers NeuN (bright) and 70 kDA Neurofilament (bright) and GFAP (dull).

Figs. 5A-F are plots and digital images showing that *in vitro* human ES cell derived neurons show histochemical, biochemical and electrophysiological features of functional mature dopaminergic neurons. **Figs. 5A-B** are graphs showing the synthesis and release of neurotransmitters measured by reverse phase HPLC (**Fig. 5A**), Levels of L-DOPA, dopamine and DOPAC in conditioned medium (stage 5, day 8). Presence of FGF8 and Shh in stage 4 significantly increase amount of catecholamines (compared to untreated controls). **Fig. 5B** shows the depolarization (50 mM KCl) leads to increased dopamine release into the cell culture medium (stage 5, day 15). **Figs. 5C-E** are graphs and digital imaging of pre-synaptic function. **Fig. 5C** shows the kinetics of synaptic release of FM4-64 dye (14 days of terminal differentiation in stage 5). Neurons were labeled with FM4-64 and serial images were taken before (*a, b*) and after (*c*) depolarization with KCl (50 mM). Depolarization induces faster release of dye from responsive synaptic puncta (arrowheads). Scale bar is 10 μm (top panel) and 5 μM (lower panel). In **Fig. 5E**, the Fluorescence ΔF/F for responsive puncta were calculated and plotted against time. Depolarization with KCl induces faster release of dye from synaptic puncta. **Fig. 5D** is a digital image of immunofluorescent staining against synapsin 1. **Fig. 5F** shows an enlarged section of a current-voltage relationship of a single cell at each stage showing the inflection points of the curve. The current voltage relations are normalized means from all traces from a single cell. Inset, Expanded current voltage relation curves (7-9 d – closed squares; 14-17 d – open circle; 22-24 d – closed triangle; 28-30 d – open diamond).

Figs. 6A-E are a set of digital images and graphs showing the functional characterization of human ES-derived dopamine neurons and the manipulation of endodermal cell fates in human EBs. **Figs. 6A-D** are digital images of

immunohistochemical staining using GATA4 (bright) and AFP (dull) as markers for endodermal precursors. Few cells positive for either GATA4 or AFP are observed in control EBs (**Fig. 6A**). Activation of wnt signaling pathway by Lithium chloride (LiCl) leads to differentiation of hypoblast (extra-embryonic endoderm, coexpression of GATA4 and AFP, **Fig. 8B**). Only regions of GATA4 positive AFP positive cells show nuclear β-catenin staining (lower inset, right panel) indicating activation of the wnt signaling pathway, whereas regions of GATA4 positive AFP negative cells show membrane bound β -catenin staining (upper inset, right panel). Serum treatment promotes formation of definitive endoderm (GATA4 positive, AFP negative), but not hypoblast (**Fig. 8C**). Quantitative analysis of LiCl treated EBs (**Fig. 7D**). **Figs. 6E-H** are digital images showing that serum treated EBs contain epithelia that coexpress Hnf3β (bright) and cdx2 (dull), which are markers for intestinal precursors (**Figs. 8E, 8F** and **8G, 8H** respectively represent same section). **Fig. 6I** is a digital image showing that, in addition, EBs contain Hnf3β (bright) positive, cdx2 (dull) negative epithelia. **Figs. 6J** and **K** are digital images showing endodermal precursors coexpress Hnf3β (dull) and Pax6 (bright, **Fig. 5K**), as well as Hnf3β (dull) and Isl1 (bright). **Figs. 6L-N** are digital images showing the differentiation of hepatocytes from endodermal precursors. **Fig. 6L** is a digital image showing Hnf3β (dull) and cytokeratin 18 (bright) positive hepatocytes. **Figs. 6M** and **6N** are digital images of enzymatic staining for γ -glutamyl-transpeptidase characteristic of fetal hepatocytes derived from LiCl (**Fig. 6M**) or serum (**Fig. 6N**) treated EBs. Inset, Hnf3β and albumin expression in hepatocytes derived from serum treated EBs. Numerous multinucleate cells are observed.

Figs. 7A-G are graphs and digital images of current clamp recordings in response to a 500 ms depolarizing current pulse (200 pA) at different time points after differentiation (14-17 d, n=18; 22-24 d, n=21; 28-30 d, n=16). **Figs. 7A-B,** development of spontaneous synaptic activity in neurons recorded in current clamp at -30 mV membrane potential for 20 second sweeps. **Fig. 7D,** enlarged section of recording at 28-30 days in culture shows spontaneous, repetitive EPSPs. **Fig. 7E,** percentage of neurons firing action potentials at each time point. **Fig. 7C**, voltage-dependent membrane currents demonstrate that depolarizing voltage steps elicit voltage-dependent inward and outward currents. **Fig. 7F**, pharmacological

perturbation indicated that the inward currents are TTX-sensitive sodium currents, while the rapidly inactivating outward currents are A-type potassium currents sensitive to 4-AP. **Fig. 7G,** biocytin-Alexa488 labeled cells (i and ii in upper and lower left panel) express TH (red) and Tuj1 (Alexa-647, false color green in lower panel) as markers for dopaminergic neurons. Right panels show corresponding recordings with voltage-dependent ionic currents and action potential generation.

Fig. 8 is a set of digital images showing the differentiation of mesoderm and germ cells from human ES cells. **Fig. 8A** shows anti-Xenopus brachyury antibody staining (green) of axial mesoderm in E7.5 mouse embryo. **Figs. 8B** and **8C** are digital images and a graph showing rachyury positive mesodermal cells after serum treatment of EBs and percentage of brachyury expressing cells in treated and untreated EBs. **Fig. 8D** is a digital image showing the time course of Brachyury mRNA induction. **Figs. 8E-H** are digital images of brachyury expressing cells in serum treated EBs overlap with Mixl (green) and Oct4 (Alexa-647, blue in **Figs. 8F-H**) expression. **Figs. 8I-N** are digital of immunohistochemical staining (at low (**Fig. 8I**) and high (**Fig. 8J**) magnification) of representative clusters of VASA (green) stained cells after BMP4 treatment. **Figs. 8K** and **8L** are a digital image of a cluster of VASA (in overlay, **Fig. 8K**, bright) and alkaline phosphatase (dull in **Fig. 8K**, **Fig. 8L**) coexpressing cells. **Figs. 8M** and **8N** are a digital image of clusters with closely associated VASA (bright) and SSEA-1 (expressing cells. **Fig. 8O** is a graph of a quantitative analysis shows significantly increased appearance of VASA stained clusters in BMP4 treated EBs compared to control, FGF4, Serum or LiCl treatment.

Figs. 9A-E is a set of digital images and plots showing the characterization of undifferentiated human ES cells and differentiation to neurectoderm. **Fig. 9A** is a digital image of immunohistochemical staining of undifferentiated human ES cells grown on mouse embryonic fibroblasts (MEF). **Fig. 9B** is a plot of a FACS analysis of undifferentiated human ES cells grown on MEF. Almost all cells express SSEA-4 (**Figs. 9A** and **9B**, left panel) and Oct-4 (**Figs. 9A** and **9B**, right panel) as markers for undifferentiated human ES cells. MEF cells are stained by anti mouse intermediate filament antibody (green). **Fig. 9C** is a digital image showing the normal karyotype of human ES cells (passage 100). **Fig. 9D** is a digital image showing increased number and altered distribution of Sox1 (left two images) and

nestin (center long images) expressing cells as markers for early neuroectodermal cells in EBs after FGF-4 (50 ng/ml) treatment (compared to untreated control EBs).

Fig. 10 is a tabular diagram showing human ES cell differentiation and culture conditions for differentiating human ES cells.

DETAILED DESCRIPTION

I. Abbreviations

AA: ascorbic acid

BDNP: brain derived neurotrophic factor

BMP: bone morphogenic protein

CNS: central nervous system

DA: dopamine

DAPI: 4',6-Diamidino-2-phenylindole

DMEM: Dulbecco's Modified Eagle's Medium

EB: embryoid body

ES: embryonic stem

FGF: fibroblast growth factor

hES: human embryonic stem cells

ICM: inner cell mass

HPLC: high performance liquid chromotography

KCl: potassium chloride

LiCL: lithium chloride

LIF: leukemia inhibitory factor

MEF: mouse embryonic fibroblasts

ml: milliliter

pmol: picomole

PNS: peripheral nervous system

RA: retinoic acid

Shh: sonic hedgehog

TH: tyrosine hydroxylase

II. Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of this disclosure, the following explanations of specific terms are provided:

Animal: Living multi-cellular vertebrate organisms, a category that includes, for example, mammals and birds. The term mammal includes both human and non-human mammals. Similarly, the term "subject" includes both human and veterinary subjects.

Central Nervous System (CNS): The part of the nervous system of an animal that contains a high concentration of cell bodies and synapses and is the main site of integration of nervous activity. In higher animals, the CNS generally refers to the brain and spinal cord.

Differentiation: The process whereby relatively unspecialized cells (e.g., embryonic cells) acquire specialized structural and/or functional features characteristic of mature cells. Similarly, "differentiate" refers to this process. Typically, during differentiation, cellular structure alters and tissue-specific proteins and properties appear. The term "differentiated neuronal cell" refers to cells expressing a protein characteristic of the specific neuronal cell type, exhibiting synaptic vesicle release, or having an electrophysiological characteristic of a neuronal cells (e.g., sustained bursts of action potentials). A differentiated neuronal cell can be a dopaminergic cell.

Differentiation medium: A synthetic set of culture conditions with the nutrients necessary to support the growth or survival of microorganisms or culture cells, and which allows the differentiation of cells, such as stem cells.

Dopaminergic neurons: Neuronal cells that produce the neurotransmitter dopamine. Typically, dopaminergic neurons are highly concentrated in the substantia nigra of the midbrain.

Dopamine, along with epinephrine, norepinephrine, and serotonin, belongs to a chemical family referred to "monoamines." Within the family of monoamines, epinephrine, norepinephrine, and dopamine are derived from the amino acid tyrosine and form a subfamily called the catecholamines. Frequently, tyrosine hydroxylase (TH), the rate-limiting enzyme for the biosynthesis of dopamine, is used as a marker to identify dopaminergic neurons.

Embryonic Stem (ES) Cells: Pluripotent cells isolated from the inner cell mass of the developing blastocyst, or the progeny of these cells. "ES cells" can be derived from any organism. ES cells can be derived from mammals, including mice, rats, rabbits, guinea pigs, goats, pigs, cows monkeys and humans. In specific, non-limiting examples, the cells are human or murine. Without being bound by theory, ES cells can generate a variety of the cells present in the body (bone, muscle, brain cells, etc.), provided they are exposed to conditions conducive to developing these cell types. Methods for producing murine ES cells can be found in U.S. Patent No. 5,670,372, which is herein incorporated by reference. Methods for producing human ES cells can be found in U.S. Patent No. 6,090,622, WO 00/70021 and WO 00/27995, which are herein incorporated by reference.

Effective amount or **Therapeutically effective amount:** The amount of agent is an amount sufficient to prevent, treat, reduce and/or ameliorate the symptoms and/or underlying causes of any disorder or disease, or the amount of an agent sufficient to produce a desired effect on a cell. In one embodiment, a "therapeutically effective amount" is an amount sufficient to reduce or eliminate a symptom of a disease. In another embodiment, a therapeutically effective amount is an amount sufficient to overcome the disease itself.

Embryoid bodies: ES cell aggregates generated when ES cells are plated on a non-adhesive surface that prevents attachment and differentiation of the ES cells. Generally, embryoid bodies include an inner core of undifferentiated stem cells surrounded by primitive endoderm.

ES proliferation media: A medium suitable for the expansion of ES cells.

Expand: A process by which the number or amount of cells in a cell culture is increased due to cell division. Similarly, the terms "expansion" or "expanded" refers to this process. The terms "proliferate," "proliferation" or "proliferated" may be used interchangeably with the words "expand," "expansion" or "expanded." Typically, during an expansion phase, the cells do not differentiate to form mature cells.

Expansion medium: A medium suitable for the expansion of CNS precursors cells. Exemplary medium components are disclosed herein.

Feeder layer: Non-proliferating cells (e.g. irradiated cells) that can be used to support proliferation of cells, including cells obtained from diverse sources including normal as well as neoplastic tissues from humans and laboratory animals. Protocols for the production of feeder layers are known in the art, and are available on the internet, such as at the National Stem Cell Resource website, which is maintained by the American Type Culture Collection (ATCC).

Fibroblast growth factor or **FGF:** Any suitable fibroblast growth factor, derived from any animal, and functional fragments thereof. A variety of FGFs are known and include, but are not limited to, FGF-1 (acidic fibroblast growth factor), FGF-2 (basic fibroblast growth factor, bFGF), FGF-3 (int-2), FGF-4 (hst/K-FGF), FGF-5, FGF-6, FGF-7, FGF-8, FGF-9 and FGF-98. "FGF" refers to a fibroblast growth factor protein such as FGF-1, FGF-2, FGF-4, FGF-6, FGF-8, FGF-9 or FGF-98, or a biologically active fragment or mutant thereof. The FGF can be from any animal species. In one embodiment, the FGF is mammalian FGF, including but not limited to, rodent, avian, canine, bovine, porcine, equine and human. The amino acid sequences and method for making many of the FGFs are well known in the art.

The amino acid sequence of human FGF-1 and a method for its recombinant expression are disclosed in U.S. Patent No. 5,604,293. The amino acid sequence of human FGF-2 and methods for its recombinant expression are disclosed in U.S. Patent No. 5,439,818, herein incorporated by reference. The amino acid sequence of bovine FGF-2 and various methods for its recombinant expression are disclosed in U.S. Patent No. 5,155,214, also herein incorporated by reference. When the 146 residue forms are compared, their amino acid sequences are nearly identical, with only two residues that differ.

The amino acid sequence of FGF-3 (Dickson et al., *Nature* 326:833, 1987) and human FGF-4 (Yoshida et al., *PHAS USA* 84:7305-7309, 1987) are known. When the amino acid sequences of human FGF-4, FGF-1, FGF-2 and murine FGF-3 are compared, residues 72-204 of human FGF-4 have 43% homology to human FGF-2; residues 79-204 have 38% homology to human FGF-1; and residues 72-174 have 40% homology to murine FGF-3. The cDNA and deduced amino acid sequences for human FGF-5 (Zhan et al., *Molec. and Cell. Biol.* 8(8):3487-3495, 1988), human FGF-6 (Coulier et al., *Oncogene* 6:1437-1444, 1991), human FGF-7 (Miyamoto et al., *Mol. and Cell. Biol.* 13(7):4251-4259, 1993) are also known. The cDNA and deduced amino acid sequence of murine FGRF-8 (Tanaka et al., *PNAS USA* 89:8928-8932, 1992), human and murine FGF-9 (Santos-Ocamp et al., *J. Biol. Chem.* 271(3):1726-1731, 1996) and human FGF-98 (provisional patent application Serial No. 60/083,553, which is hereby incorporated herein by reference in its entirety) are also known.

FGF-2 (also known as bFGF or bFGF-2), and other FGFs, can be made as described in U.S. Patent No. 5,155,214 ("the '214 patent"). The recombinant bFGF-2, and other FGFs, can be purified to pharmaceutical quality (98% or greater purity) using the techniques described in detail in U.S. Patent No. 4,956,455.

FGF-4 is the product of the hst oncogene (also known as hst-1 or hst). The amino acid sequence for human FGF-4 was first disclosed by Yoshida et al., *Proc. Natl. Acad. Sci. USA* 84:7305-7309, 1987, at Fig. 3. The endogenous human protein encoded has a molecular mass of 23 kDa. FGF-4 has been implicated recently as one of the molecules that directs outgrowth and patterning of the limb during chick embryonic growth (see Adelaide et al., *Oncogene* 2:413-416, 1988; see also U.S. Patent No. 6,277,820).

Fibroblast growth factor-8 (FGF-8), alternatively known as androgen-induced growth factor (AIGF) is a member of the FGF family known to influence embryogenesis and morphogenesis. The *in situ* embryonic expression pattern suggests a unique role of FGF-8 in mouse development, especially in gastrulation, brain development, and limb and facial morphogenesis. Ohuchi et al., *Biochem. Biophys. Res. Commun.* 204(2):882-888, 1994. Northern blot expression reveals a unique temporal and spatial pattern of FGF-8 expression in the developing mouse

and suggests a role for this FGF in multiple regions of ectodermal differentiation in the post-gastrulation mouse embryo. Heikinheimo et al., *Mech. Dev.* 48(2):129-138, 1994. A sequence of FGF-8 is shown in U.S. Patent No. 6,277,820.

Biologically active variants of FGF are also of use with the methods disclosed herein. Such variants should retain FGF activities, particularly the ability to bind to FGF receptor sites. FGF activity may be measured using standard FGF bioassays, which are known to those of skill in the art. Representative assays include known radioreceptor assays using membranes, a bioassay that measures the ability of the molecule to enhance incorporation of tritiated thymidine, in a dose-dependent manner, into the DNA of cells, and the like. Preferably, the variant has at least the same activity as the native molecule.

In addition to the above described FGFs, an agent of use also includes an active fragment of any one of the above-described FGFs. In its simplest form, the active fragment is made by the removal of the N-terminal methionine, using well-known techniques for N-terminal methionine removal, such as a treatment with a methionine aminopeptidase. A second desirable truncation includes an FGF without its leader sequence. Those skilled in the art recognize the leader sequence as the series of hydrophobic residues at the N-terminus of a protein that facilitate its passage through a cell membrane but that are not necessary for activity and that are not found on the mature protein.

Preferred truncations on the FGFs are determined relative to mature FGF-2 having 146 residues. As a general rule, the amino acid sequence of an FGF is aligned with FGF-2 to obtain maximum homology. Portions of the FGF that extend beyond the corresponding N-terminus of the aligned FGF-2 are generally suitable for deletion without adverse effect. Likewise, portions of the FGF that extend beyond the C-terminus of the aligned FGF-2 are also capable of being deleted without adverse effect.

Fragments of FGF that are smaller than those described can also be employed in the present methods. It should be noted that human and murine FGF-2, FGF-4, FGF-8 and a variety of other FGFs, are commercially available.

Suitable biologically active variants can be FGF analogs or derivatives. By "analog" is intended an analog of either FGF or an FGF fragment that includes a

native FGF sequence and structure having one or more amino acid substitutions, insertions, or deletions. Analogs having one or more peptoid sequences (peptide mimic sequences) are also included (see e.g. International Publication No. WO 91/04282). By "derivative" is intended any suitable modification of FGF, FGF fragments, or their respective analogs, such as glycosylation, phosphorylation, or other addition of foreign moieties, as long as the FGF activity is retained. Methods for making FGF fragments, analogs and derivatives are available in the art.

In addition to the above-described FGFs, the methods disclosed herein can also employ an active mutant or variant thereof. By the term active mutant, as used in conjunction with an FGF, is meant a mutated form of the naturally occurring FGF. FGF mutant or variants will generally have at least 70%, preferably 80%, more preferably 85%, even more preferably 90% to 95% or more, and for example 98% or more amino acid sequence identity to the amino acid sequence of the reference FGF molecule. A mutant or variant may, for example, differ by as few as 1 to 10 amino acid residues, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The sequence identity can be determined as described herein. For FGF, one method for determining sequence identify employs the Smith-Waterman homology search algorithm (*Meth. Mol. Biol.* 70:173-187, 1997) as implemented in MSPRCH program (Oxford Molecular) using an affine gap search with the following search parameters: gap open penalty of 12, and gap extension penalty of 1. In one embodiment, the mutations are "conservative amino acid substitutions" using L-amino acids, wherein one amino acid is replaced by another biologically similar amino acid. Conservative amino acid substitutions are those that preserve the general charge, hydrophobicity, hydrophilicity, and/or steric bulk of the amino acid being substituted.

One skilled in the art, using art known techniques, is able to make one or more point mutations in the DNA encoding any of the FGFs to obtain expression of an FGF polypeptide mutant (or fragment mutant) having an activity for use in methods disclosed herein. To prepare a biologically active mutant of an FGF, one uses standard techniques for site directed mutagenesis, as known in the art and/or as

taught in Gilman et al., *Gene* 8:81, 1979 or Roberts et al., *Nature* 328:731, 1987, to introduce one or more point mutations into the cDNA that encodes the FGF.

Growth factor: A substance that promotes cell growth, survival, and/or differentiation. Growth factors include molecules that function as growth stimulators (mitogens), molecules that function as growth inhibitors (e.g. negative growth factors) factors that stimulate cell migration, factors that function as chemotactic agents or inhibit cell migration or invasion of tumor cells, factors that modulate differentiated functions of cells, factors involved in apoptosis, or factors that promote survival of cells without influencing growth and differentiation. Examples of growth factors are bFGF, epidermal growth factor (EGF), CNTF, HGF, nerve growth factor (NGF), and actvin-A.

Growth medium or **expansion medium:** A synthetic set of culture conditions with the nutrients necessary to support the growth (cell division/expansion) of a specific population of cells. In one embodiment, the cells are ES cells. In this embodiment, the growth media is an ES growth medium that allows ES cells to proliferate. In another embodiment, the cells are neuronal precursor cells. In this embodiment, the expansion medium is a neuronal precursor cell expansion medium that allows neuronal precursors to proliferate.

Growth media generally include a carbon source, a nitrogen source and a buffer to maintain pH. In one embodiment, ES growth medium contains a minimal essential media, such as DMEM, supplemented with various nutrients to enhance ES cell growth. Additionally, the minimal essential media may be supplemented with additives such as horse, calf or fetal bovine serum.

Heterologous: A heterologous sequence is a sequence that is not normally (i.e. in the wild-type sequence) found adjacent to a second sequence. In one embodiment, the sequence is from a different genetic source, such as a virus or organism, than the second sequence.

Hybridization: A process wherein oligonucleotides and their analogs bind by hydrogen bonding, which includes Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary bases. Generally, nucleic acid consists of nitrogenous bases that are either pyrimidines (Cytosine (C), uracil (U), and thymine(T)) or purines (adenine (A) and guanine (G)). These nitrogenous

bases form hydrogen bonds consisting of a pyrimidine bonded to a purine, and the bonding of the pyrimidine to the purine is referred to as "base pairing." More specifically, A will bond to T or U, and G will bond to C. "Complementary" refers to the base pairing that occurs between two distinct nucleic acid sequences or two distinct regions of the same nucleic acid sequence. For example, a M-CSF antagonist can be an oligonucleotide complementary to a M-CSF encoding mRNA, or a M-CSF encoding dsDNA.

"Specifically hybridizable" and "specifically complementary" are terms which indicate a sufficient degree of complementarity such that stable and specific binding occurs between the oligonucleotide (or its analog) and the DNA or RNA target. The oligonucleotide or oligonucleotide analog need not be 100% complementary to its target sequence to be specifically hybridizable. An oligonucleotide or analog is specifically hybridizable when binding of the oligonucleotide or analog to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide or analog to non-target sequences under conditions in which specific binding is desired, for example under physiological conditions in the case of *in vivo* assays. Such binding is referred to as "specific hybridization."

Hybridization conditions resulting in particular degrees of stringency will vary depending upon the nature of the hybridization method of choice and the composition and length of the hybridizing nucleic acid sequences. Generally, the temperature of hybridization and the ionic strength (especially the Na^+ concentration) of the hybridization buffer will determine the stringency of hybridization.

Nucleic acid duplex or hybrid stability is expressed as the melting temperature or Tm, which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions. If sequences are to be identified that are related and substantially identical to the probe, rather than identical, then it is useful to first establish the lowest temperature at which only homologous hybridization occurs with a particular concentration of salt (e.g., SSC or SSPE). Then, assuming that 1% mismatching

results in a 1°C decrease in the Tm, the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having >95% identity with the probe are sought, the final wash temperature is decreased by 5°C). In practice, the change in Tm can be between 0.5°C and 1.5°C per 1% mismatch. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed by Sambrook et al. (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, chapters 9 and 11, herein incorporated by reference.

For purposes of the present invention, "stringent conditions" encompass conditions under which hybridization will only occur if there is less than 30% mismatch between the hybridization molecule and the target sequence. "Stringent conditions" may be broken down into particular levels of stringency for more precise definition. Thus, as used herein, "moderate stringency" conditions are those under which molecules with more than 30% sequence mismatch will not hybridize; conditions of "medium stringency" are those under which molecules with more than 20% mismatch will not hybridize, and conditions of "high stringency" are those under which sequences with more than 10% mismatch will not hybridize.

Molecules with complementary nucleic acids form a stable duplex or triplex when the strands bind, or hybridize, to each other by forming Watson-Crick, Hoogsteen or reverse Hoogsteen base pairs. Stable binding occurs when an oligonucleotide remains detectably bound to a target nucleic acid sequence under the required conditions. "Complementarity" is the degree to which bases in one nucleic acid strand base pair with the bases in a second nucleic acid strand. Complementarity is conveniently described by the percentage, i.e. the proportion of nucleotides that form base pairs between two strands or within a specific region or domain of two strands. For example, if 10 nucleotides of a oligonucleotide of 15 nucleotides in length form base pairs with a targeted region of a DNA molecule, that oligonucleotide is said to have 66.67% complementarity to the region of DNA targeted.

In the present disclosure, "sufficient complementarity" means that a sufficient number of base pairs exist between the oligonucleotide and the target sequence to achieve detectable binding, and disrupt expression of gene products (such as FGF-2, FGF-4 or FGF-8). When expressed or measured by percentage of base pairs formed, the percentage complementarity that fulfills this goal can range from as little as about 50% complementarity to full, (100%) complementarity. In general, sufficient complementarity is at least about 50%. In one embodiment, sufficient complementarity is at least about 75% complementarity. In another embodiment, sufficient complementarity is at least about 90% or about 95% complementarity. In yet another embodiment, sufficient complementarity is at least about 98% or 100% complementarity.

A thorough treatment of the qualitative and quantitative considerations involved in establishing binding conditions that allow one skilled in the art to design appropriate oligonucleotides for use under the desired conditions is provided by Beltz et al., *Methods Enzymol* 100:266-285, 1983, and by Sambrook et al. (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Isolated: An "isolated" biological component (such as a nucleic acid, peptide or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids, peptides and proteins which have been "isolated" thus include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids.

LIF (Leukemia Inhibitory Factor): A growth factor that prevents differentiation of ES cells. LIF is a heavily and variably glycosylated 58 kDa protein with a length of 179 amino acids. Glycosylation does not appear to be essential for bioactivity. Two different glycosylation variants have been designated as LIF-A and LIF-B. The murine and human factors show a homology of 79% at

the amino acid level. Both factors show a high degree of conservative amino acid exchanges.

Neurological disorder: A disorder in the nervous system, including the central nervous system (CNS) and peripheral nervous system (PNS). Examples of neurological disorders include Parkinson's disease, Huntington's disease, Alzheimer's disease, severe seizure disorders including epilepsy, familial dysautonomia as well as injury or trauma to the nervous system, such as neurotoxic injury or disorders of mood and behavior such as addiction, schizophrenia and amyotrophic lateral sclerosis. Neuronal disorders also include Lewy body dementia, multiple sclerosis, epilepsy, cerebellar ataxia, progressive supranuclear palsy, amyotrophic lateral sclerosis, affective disorders, anxiety disorders, obsessive compulsive disorders, personality disorders, attention deficit disorder, attention deficit hyperactivity disorder, Tourette Syndrome, Tay Sachs, Nieman Pick, and other lipid storage and genetic brain diseases and/or schizophrenia

Neurodegenerative disorder: An abnormality in the nervous system of a subject, such as a mammal, in which neuronal integrity is threatened. Without being bound by theory, neuronal integrity can be threatened when neuronal cells display decreased survival or when the neurons can no longer propagate a signal. Specific, non-limiting examples of a neurodegenerative disorder are Alzheimer's disease, Pantothenate kinase associated neurodegeneration, Parkinson's disease, Huntington's disease (Dexter et al., *Brain* 114:1953-1975, 1991), HIV encephalopathy (Miszkziel et al., *Magnetic Res. Imag.* 15:1113-1119, 1997), and amyotrophic lateral sclerosis.

Alzheimer's disease manifests itself as pre-senile dementia. The disease is characterized by confusion, memory failure, disorientation, restlessness, speech disturbances, and hallucination in mammals (*Medical, Nursing, and Allied Health Dictionary*, 4th Ed., 1994, Editors: Anderson, Anderson, Glanze, St. Louis, Mosby).

Parkinson's disease is a slowly progressive, degenerative, neurologic disorder characterized by resting tremor, loss of postural reflexes, and muscle rigidity and weakness (*Medical, Nursing, and Allied Health Dictionary*, 4th Ed., 1994, Editors: Anderson, Anderson, Glanze, St. Louis, Mosby).

Amyotrophic lateral sclerosis is a degenerative disease of the motor neurons characterized by weakness and atrophy of the muscles of the hands, forearms and legs, spreading to involve most of the body and face (*Medical, Nursing, and Allied Health Dictionary*, 4th Ed., 1994, Editors: Anderson, Anderson, Glanze, St. Louis, Mosby).

Pantothenate kinase associated neurodegeneration (PKAN, also known as Hallervorden-Spatz syndrome) is an autosomal recessive neurodegenerative disorder associated with brain iron accumulation. Clinical features include extrapyramidal dysfunction, onset in childhood, and a relentlessly progressive course (Dooling et al., *Arch. Neurol.* 30:70-83, 1974). PKAN is a clinically heterogeneous group of disorders that includes classical disease with onset in the first two decades, dystonia, high globus pallidus iron with a characteristic radiographic appearance (Angelini et al., *J. Neurol.* 239:417-425, 1992), and often either pigmentary retinopathy or optic atrophy (Dooling et al., *Arch. Neurol.* 30:70-83, 1974; Swaiman et al., *Arch. Neurol* 48:1285-1293, 1991).

A "neurodegenerative related disorder" is a disorder such as speech disorders that are associated with a neurodegenerative disorder. Specific non-limiting examples of a neurodegenerative related disorders include, but are not limited to, palilalia, tachylalia, echolalia, gait disturbance, perseverative movements, bradykinesia, spasticity, rigidity, retinopathy, optic atrophy, dysarthria, and dementia.

Nucleotide: A monomer that includes a base linked to a sugar, such as a pyrimidine, purine or synthetic analogs thereof, or a base linked to an amino acid, as in a peptide nucleic acid (PNA). A nucleotide is one monomer in a polynucleotide. A nucleotide sequence refers to the sequence of bases in a polynucleotide.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

Peripheral Nervous System (PNS): The part of an animal's nervous system other than the Central Nervous System. Generally, the PNS is located in the peripheral parts of the body and includes cranial nerves, spinal nerves and their branches, and the autonomic nervous system.

Polypeptide: A polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being preferred. The terms "polypeptide" or "protein" as used herein is intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. The term "polypeptide" is specifically intended to cover naturally occurring proteins, as well as those which are recombinantly or synthetically produced.

The term "polypeptide fragment" refers to a portion of a polypeptide which exhibits at least one useful epitope. The term "functional fragments of a polypeptide" refers to all fragments of a polypeptide that retain an activity of the polypeptide. Biologically functional fragments, for example, can vary in size from a polypeptide fragment as small as an epitope capable of binding an antibody molecule to a large polypeptide capable of participating in the characteristic induction or programming of phenotypic changes within a cell. An "epitope" is a region of a polypeptide capable of binding an immunoglobulin generated in response to contact with an antigen. Thus, smaller peptides containing the biological activity of insulin, or conservative variants of the insulin, are thus included as being of use.

The term "soluble" refers to a form of a polypeptide that is not inserted into a cell membrane.

The term "substantially purified polypeptide" as used herein refers to a polypeptide which is substantially free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. In one embodiment, the polypeptide is at least 50%, for example at least 80% free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. In another embodiment, the polypeptide is at least 90% free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated. In yet another

embodiment, the polypeptide is at least 95% free of other proteins, lipids, carbohydrates or other materials with which it is naturally associated.

Conservative substitutions replace one amino acid with another amino acid that is similar in size, hydrophobicity, etc. Examples of conservative substitutions are shown below.

Original Residue	Conservative Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu, Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Variations in the cDNA sequence that result in amino acid changes, whether conservative or not, should be minimized in order to preserve the functional and immunologic identity of the encoded protein. The immunologic identity of the protein may be assessed by determining whether it is recognized by an antibody; a variant that is recognized by such an antibody is immunologically conserved. Any cDNA sequence variant will preferably introduce no more than twenty, and preferably fewer than ten amino acid substitutions into the encoded polypeptide. Variant amino acid sequences may, for example, be 80%, 90% or even 95% or 98% identical to the native amino acid sequence.

Pharmaceutically acceptable carriers: The pharmaceutically acceptable carriers useful in this invention are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of the fusion proteins herein disclosed.

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (e.g., powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Pharmaceutical agent or **"drug":** A chemical compound or composition capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject or a cell. "Incubating" includes a sufficient amount of time for a drug to interact with a cell. "Contacting" includes incubating a drug in solid or in liquid form with a cell.

Polynucleotide: A nucleic acid sequence (such as a linear sequence) of any length. Therefore, a polynucleotide includes oligonucleotides, and also gene sequences found in chromosomes. An "oligonucleotide" is a plurality of joined nucleotides joined by native phosphodiester bonds. An oligonucleotide is a polynucleotide of between 6 and 300 nucleotides in length. An oligonucleotide analog refers to moieties that function similarly to oligonucleotides but have non-naturally occurring portions. For example, oligonucleotide analogs can contain non-naturally occurring portions, such as altered sugar moieties or inter-sugar linkages, such as a phosphorothioate oligodeoxynucleotide. Functional analogs of naturally occurring polynucleotides can bind to RNA or DNA, and include peptide nucleic acid (PNA) molecules.

Primers: Short nucleic acids, for example DNA oligonucleotides 10 nucleotides or more in length, which are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a DNA polymerase enzyme. Primer pairs can be used for amplification of a nucleic acid

sequence, e.g., by the polymerase chain reaction (PCR) or other nucleic-acid amplification methods known in the art.

Probes and primers as used in the present disclosure may, for example, include at least 10 nucleotides of the nucleic acid sequences that are shown to encode specific proteins. In order to enhance specificity, longer probes and primers may also be employed, such as probes and primers that comprise 15, 20, 30, 40, 50, 60, 70, 80, 90 or 100 consecutive nucleotides of the disclosed nucleic acid sequences. Methods for preparing and using probes and primers are described in the references, for example Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York; Ausubel et al. (1987) *Current Protocols in Molecular Biology*, Greene Publ. Assoc. & Wiley-Intersciences; Innis et al. (1990) *PCR Protocols, A Guide to Methods and Applications*, Innis et al. (Eds.), Academic Press, San Diego, CA. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, 1991, Whitehead Institute for Biomedical Research, Cambridge, MA).

When referring to a probe or primer, the term *specific for (a target sequence)* indicates that the probe or primer hybridizes under stringent conditions substantially only to the target sequence in a given sample comprising the target sequence.

Promoter: A promoter is an array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Similarly, a recombinant protein is one coded for by a recombinant nucleic acid molecule.

Sequence identity: The similarity between amino acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or variants of a FGF polypeptide will possess a relatively high degree of sequence identity when aligned using standard methods.

Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in Smith and Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins and Sharp, *Gene* 73:237, 1988; Higgins and Sharp, *CABIOS* 5:151, 1989; Corpet et al., *Nucleic Acids Research* 16:10881, 1988; and Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988. Altschul, et al., *Nature Genet.*, 6:119, 1994 presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul, et al., *J. Mol. Biol.* 215:403, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. A description of how to determine sequence identity using this program is available on the NCBI website on the internet.

Homologs and variants of a FGF polypeptide are typically characterized by possession of at least about 75%, for example at least about 80%, sequence identity counted over the full length alignment with the amino acid sequence of the factor using the NCBI Blast 2.0, gapped blastp set to default parameters. For comparisons of amino acid sequences of greater than about 30 amino acids, the Blast 2 sequences function is employed using the default BLOSUM62 matrix set to default parameters, (gap existence cost of 11, and a per residue gap cost of 1). When aligning short peptides (fewer than around 30 amino acids), the alignment should be performed using the Blast 2 sequences function, employing the PAM30 matrix set to default parameters (open gap 9, extension gap 1 penalties). Proteins with even greater similarity to the reference sequences will show increasing percentage identities when assessed by this method, such as at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or

at least 99% sequence identity. When less than the entire sequence is being compared for sequence identity, homologs and variants will typically possess at least 80% sequence identity over short windows of 10-20 amino acids, and may possess sequence identities of at least 85% or at least 90% or 95% depending on their similarity to the reference sequence. Methods for determining sequence identity over such short windows are available at the NCBI website on the internet. One of skill in the art will appreciate that these sequence identity ranges are provided for guidance only; it is entirely possible that strongly significant homologs could be obtained that fall outside of the ranges provided.

Sonic hedgehog (Shh): Sonic hedgehog (Shh) is one of three mammalian homologs of the Drosophila hedgehog signaling molecule and is expressed at high levels in the notochord and floor plate of developing embryos. Shh is known to play a key role in neuronal tube patterning (Echerlard et al., *Cell* 75:1417-30, 1993), the development of limbs, somites, lungs and skin. Moreover, overexpression of Shh has been found in basal cell carcinoma. A sequence of Shh is set forth is U.S. Patent No. 6,277,820.

Stem cell: A cell that can generate a fully differentiated functional cell of more than one given cell type. The role of stem cells *in vivo* is to replace cells that are destroyed during the normal life of an animal. Generally, stem cells can divide without limit. After division, the stem cell may remain as a stem cell, become a precursor cell, or proceed to terminal differentiation. Although appearing morphologically unspecialized, the stem cell may be considered differentiated where the possibilities for further differentiation are limited. A precursor cell is a cell that can generate a fully differentiated functional cell of at least one given cell type. Generally, precursor cells can divide. After division, a precursor cell can remain a precursor cell, or may proceed to terminal differentiation. A "neuronal stem cell" is a stem cell that can be differentiated into neurons. In one embodiment, a neuronal stem cell gives rise to all of the types of neuronal cells (e.g. dopaminergic, adrenergic, and serotinergic neurons) but does not give rise to other cells such as glial cells. A "neuronal precursor cell" is a precursor cell of the nervous system. In one embodiment, a pancreatic precursor cell gives rise to more than one type of

neuron. One example of a neuronal precursor cells is a cell that gives rise to dopaminergic neurons.

Subject: Any mammal, such as humans, non-human primates, pigs, sheep, cows, rodents and the like which is to be the recipient of the particular treatment. In one embodiment, a subject is a human subject or a murine subject.

Synapse: Highly specialized intercellular junctions between neurons and between neurons and effector cells across which a nerve impulse is conducted (synaptically active). Generally, the nerve impulse is conducted by the release from one neuron (presynaptic neuron) of a chemical transmitter (such as dopamine or serotonin) which diffuses across the narrow intercellular space to the other neuron or effector cell (post-synaptic neuron). Generally neurotransmitters mediate their effects by interacting with specific receptors incorporated in the post-synaptic cell. "Synaptically active" refers to cells (e.g., differentiated neurons) which receive and transmit action potentials characteristic of mature neurons.

Therapeutic agent: Used in a generic sense, it includes treating agents, prophylactic agents, and replacement agents.

Transduced, Transformed and **Transfected:** A virus or vector "transduces" a cell when it transfers nucleic acid into the cell. A cell is "transformed" or "transfected" by a nucleic acid transduced into the cell when the DNA becomes stably replicated by the cell, either by incorporation of the nucleic acid into the cellular genome, or by episomal replication.

Numerous methods of transfection are known to those skilled in the art, such as: chemical methods (e.g., calcium-phosphate transfection), physical methods (e.g., electroporation, microinjection, particle bombardment), fusion (e.g., liposomes), receptor-mediated endocytosis (e.g., DNA-protein complexes, viral envelope/capsid-DNA complexes) and by biological infection by viruses such as recombinant viruses (Wolff, J. A., ed, *Gene Therapeutics*, Birkhauser, Boston, USA, 1994). In the case of infection by retroviruses, the infecting retrovirus particles are absorbed by the target cells, resulting in reverse transcription of the retroviral RNA genome and integration of the resulting provirus into the cellular DNA. Methods for the introduction of genes into the pancreatic endocrine cells are known (e.g. see U.S. Patent No. 6,110,743, herein incorporated by reference). These methods can be used

to transduce a pancreatic endocrine cell produced by the methods described herein, or an artificial islet produced by the methods described herein.

Genetic modification of the target cell is an indicium of successful transfection. "Genetically modified cells" refers to cells whose genotypes have been altered as a result of cellular uptakes of exogenous nucleotide sequence by transfection. A reference to a transfected cell or a genetically modified cell includes both the particular cell into which a vector or polynucleotide is introduced and progeny of that cell.

Transgene: An exogenous gene supplied by a vector.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication. A vector may also include one or more therapeutic genes and/or selectable marker genes and other genetic elements known in the art. A vector can transduce, transform or infect a cell, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell. A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The term "comprises" means "includes." All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including explanations of terms, will

control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Method for Differentiating Embryonic Stem (ES) Cells

ES cells can proliferate indefinitely in an undifferentiated state. Furthermore, ES cells are totipotent cells, meaning that they can generate all of the cells present in the body (bone, muscle, brain cells, etc.). ES cells have been isolated from the inner cell mass (ICM) of the developing murine blastocyst (Evans et al., *Nature* 292:154-156, 1981; Martin et al., *Proc. Natl. Acad. Sci.* 78:7634-7636, 1981; Robertson et al., *Nature* 323:445-448, 1986). Additionally, human cells with ES properties have been isolated from the inner blastocyst cell mass (Thomson et al., *Science* 282:1145-1147, 1998) and developing germ cells (Shamblott et al., *Proc. Natl. Acad. Sci. USA* 95:13726-13731, 1998), and human and non-human primate embryonic stem cells have been produced (see U.S. Patent No. 6,200,806, which is incorporated by reference herein).

The methods and cells described herein are based on the discovery that embryonic stem cells can be differentiated *in vitro* to form any tissue of interest. Thus, embryonic stem cells can be differentiated to form neuronal cells, such as dopaminergic cells. Embryonic stem cells can also be differentiated into endodermal cells, such as hepatocytes. In one embodiment, the cells are of primate origin, such as human origin. The cells can also be of non-human primate origin.

As disclosed in U.S. Patent No. 6,200,806, ES cells can be produced from human and non-human primates. In one embodiment, primate ES cells are isolated "ES medium" that express SSEA-3; SSEA-4, TRA-1-60, and TRA-1-81 (see U.S. Patent No. 6,200,806). ES medium consists of 80% Dulbecco's modified Eagle's medium (DMEM; no pyruvate, high glucose formulation, Gibco BRL), with 20% fetal bovine serum (FBS; Hyclone), 0.1 mM ß-mercaptoethanol (Sigma), 1% non-essential amino acid stock (Gibco BRL). Generally, primate ES cells are isolated on a confluent layer of murine embryonic fibroblast in the presence of ES cell medium. In one example, embryonic fibroblasts are obtained from 12 day old fetuses from outbred mice (such as CF1, available from SASCO), but other strains may be used as an alternative. Tissue culture dishes treated with 0.1% gelatin (type I; Sigma) can

be utilized. Distinguishing features of ES cells, as compared to the committed "multipotential" stem cells present in adults, include the capacity of ES cells to maintain an undifferentiated state indefinitely in culture, and the potential that ES cells have to develop into every different cell types. Unlike mouse ES cells, human ES (hES) cells do not express the stage-specific embryonic antigen SSEA-1, but express SSEA-4, which is another glycolipid cell surface antigen recognized by a specific monoclonal antibody (see, e.g., Amit et al., *Devel. Biol.* 227:271-278, 2000,

For rhesus monkey embryos, adult female rhesus monkeys (greater than four years old) demonstrating normal ovarian cycles are observed daily for evidence of menstrual bleeding (day 1 of cycle=the day of onset of menses). Blood samples are drawn daily during the follicular phase starting from day 8 of the menstrual cycle, and serum concentrations of luteinizing hormone are determined by radioimmunoassay. The female is paired with a male rhesus monkey of proven fertility from day 9 of the menstrual cycle until 48 hours after the luteinizing hormone surge; ovulation is taken as the day following the luteinizing hormone surge. Expanded blastocysts are collected by non-surgical uterine flushing at six days after ovulation. This procedure generally results in the recovery of an average 0.4 to 0.6 viable embryos per rhesus monkey per month (Seshagiri et al., *Am J Primatol.* 29:81-91, 1993).

For marmoset embryos, adult female marmosets (greater than two years of age) demonstrating regular ovarian cycles are maintained in family groups, with a fertile male and up to five progeny. Ovarian cycles are controlled by intramuscular injection of 0.75 g of the prostaglandin PGF2a analog cloprostenol (Estrumate, Mobay Corp, Shawnee, KS) during the middle to late luteal phase. Blood samples are drawn on day 0 (immediately before cloprostenol injection), and on days 3, 7, 9, 11, and 13. Plasma progesterone concentrations are determined by ELISA. The day of ovulation is taken as the day preceding a plasma progesterone concentration of 10 ng/ml or more. At eight days after ovulation, expanded blastocysts are recovered by a non-surgical uterine flush procedure (Thomson et al., *J Med Primatol.* 23:333-336, 1994). This procedure results in the average production of 1.0 viable embryos per marmoset per month.

The zona pellucida is removed from blastocysts, such as by brief exposure to pronase (Sigma). For immunosurgery, blastocysts are exposed to a 1:50 dilution of rabbit anti-marmoset spleen cell antiserum (for marmoset blastocysts) or a 1:50 dilution of rabbit anti-rhesus monkey (for rhesus monkey blastocysts) in DMEM for 30 minutes, then washed for 5 minutes three times in DMEM, then exposed to a 1:5 dilution of Guinea pig complement (Gibco) for 3 minutes. After two further washes in DMEM, lysed trophectoderm cells are removed from the intact inner cell mass (ICM) by gentle pipetting, and the ICM plated on mouse inactivated (3000 rads gamma irradiation) embryonic fibroblasts.

After 7-21 days, ICM-derived masses are removed from endoderm outgrowths with a micropipette with direct observation under a stereo microscope, exposed to 0.05% Trypsin-EDTA (Gibco) supplemented with 1% chicken serum for 3-5 minutes and gently dissociated by gentle pipetting through a flame polished micropipette.

Dissociated cells are re-plated on embryonic feeder layers in fresh ES medium, and observed for colony formation. Colonies demonstrating ES-like morphology are individually selected, and split again as described above. The ES-like morphology is defined as compact colonies having a high nucleus to cytoplasm ratio and prominent nucleoli. Resulting ES cells are then routinely split by brief trypsinization or exposure to Dulbecco's Phosphate Buffered Saline (PBS, without calcium or magnesium and with 2 mM EDTA) every 1-2 weeks as the cultures become dense. Early passage cells are also frozen and stored in liquid nitrogen.

Cell lines may be karyotyped with a standard G-banding technique (such as by the Cytogenetics Laboratory of the University of Wisconsin State Hygiene Laboratory, which provides routine karyotyping services) and compared to published karyotypes for the primate species.

Isolation of ES cell lines from other primate species would follow a similar procedure, except that the rate of development to blastocyst can vary by a few days between species, and the rate of development of the cultured ICMs will vary between species. For example, six days after ovulation, rhesus monkey embryos are at the expanded blastocyst stage, whereas marmoset embryos do not reach the same

stage until 7-8 days after ovulation. The rhesus ES cell lines can be obtained by splitting the ICM-derived cells for the first time at 7-16 days after immunosurgery; whereas the marmoset ES cells were derived with the initial split at 7-10 days after immunosurgery. Because other primates also vary in their developmental rate, the timing of embryo collection, and the timing of the initial ICM split, varies between primate species, but the same techniques and culture conditions will allow ES cell isolation (see U.S. Patent No. 6, 200,806, which is incorporated herein by reference for a complete discussion of primate ES cells and their production).

Human ES cell lines exist and can be used in the methods disclosed herein. Human ES cells can also be derived from preimplantation embryos from *in vitro* fertilized (IVF) embryos. Experiments on unused human IVF-produced embryos are allowed in many countries, such as Singapore and the United Kingdom, if the embryos are less than 14 days old. Only high quality embryos are suitable for ES isolation. Present defined culture conditions for culturing the one cell human embryo to the expanded blastocyst have been described (see Bongso et al., *Hum Reprod.* 4:706-713, 1989). Co-culturing of human embryos with human oviductal cells results in the production of high blastocyst quality. IVF-derived expanded human blastocysts grown in cellular co-culture, or in improved defined medium, allows isolation of human ES cells with the same procedures described above for non-human primates (see U.S. Patent No. 6,200,806).

In one specific, non-limiting example, the method includes generating embryoid bodies from a culture of undifferentiated embryonic stem cells, selecting neuronal precursor cells, expanding the neuronal precursor cells by culturing endocrine cells in an expansion medium that comprises a growth factor and differentiating the expanded neuronal precursor cells in a differentiation media to differentiated neuronal cells. An example of this method is outlined below.

In another specific, non limiting example, the method includes culturing a monolayer of embryonic stem cells in a media to expand the cells.

Multi-Step Method for Differentiating Human Embryonic Stem Cells into Neuronal Cells

Disclosed herein are methods for generating neuronal cells from primate embryonic stem (ES) cells, including both human and non-human primate ES cells using a multi-step method. Disclosed are methods to produce neural precursor cells, glial cells, dopaminergic cells, sertoinergic cells, and motor neurons. These methods are described in detail below, and in the accompanying examples.

Expansion of undifferentiated embryonic stem (ES) cells

The expansion of primate ES cells prior to differentiation is not required to perform the method disclosed herein. However, to increase the number of neuronal cells formed, ES cells can be expanded prior to embryoid body formation. Undifferentiated embryonic stem (ES) cells are cultured in ES proliferation media to expand the number of cells. Without being bound by theory, it is believed that primate ES cells can be expanded at least about 1000 fold without losing pluripotency. In one embodiment, the ES cells are human ES cells such as H9.1 or H9.1 (Amit et al., *Devel. Bio.* 227: 271-8, 2000; Thomson et al., *Science* 282, 5391, 1998) or human embryonic germ cells (EG cells) (Shamblot et al., *Proc. Natl. Acad. Sci. USA* 95:13726, 1998). Any human or non-human primate ES cell can be utilized with the methods disclosed herein (see U.S. Patent No. 6,200,806, which is incorporated by reference in its entirety).

The ES cells are cultured in an ES growth medium which generally includes a carbon source, a nitrogen source and a buffer to maintain pH. In one embodiment, ES growth medium contains a minimal essential medium, such as Dulbecco's Modified Eagle's Medium (DMEM), supplemented with various nutrients to enhance ES cell growth. Additionally, the minimal essential medium may be supplemented with additives such as horse, calf or fetal bovine serum (for example, from between about 10% by volume to about 20% by volume or about 15% by volume) and may be supplemental with nonessential amino acids, L-glutamine, and antibiotics such as streptomycin, penicillin, and combinations thereof. In addition, 2-mercaptoethanol may also be included in the media. ES growth media is

commercially available, for example as KO-DMEM (Life-Tech Catalog No. 10829-018).

Other methods and media for obtaining and culturing embryonic stem cells are known and are suitable for use (Evans et al., *Nature* 292:154-156, 1981; Martin et al., *Proc. Natl. Acad. Sci.* 78:7634-7636, 1981; Robertson et al., *Nature* 323:445-448, 1986; Doetschman et al., *Nature* 330:576-578, 1987; Thomas et al., *Cell* 51:503-512, 1987; Thomson et al., *Science* 282:1145-1147, 1998; and Shamblott et al., *Proc. Natl. Acad. Sci. USA* 95:13726-13731, 1998). The disclosures of these references are incorporated by reference herein.

In one specific, non-limiting example, the ES cells are cultured on plates which prevent differentiation of the ES cells. Suitable plates include those such as gelatin coated tissue culture plates, or plates which include a feeder cell layer. "Feeder cells" or "feeders" are terms used to describe cells of one tissue type that are co-cultured with cells of a tissue type, to provide an environment in which the cells of the second tissue type can grow. The feeder cells are optionally from a different species as the cells they are supporting. For example, certain types of ES cells can be supported by primary cultures of mouse embryonic fibroblasts, immortalized mouse embryonic fibroblasts, as described in U.S. Patent No. 6,642,048. In co-culture with ES cells, feeder cells are typically inactivated by irradiation or treatment with an anti-mitotic agent such as mitomycin c, to prevent them from outgrowing the cells they are supporting. Feeder layers of used include a fibroblast feeder cell layer, such as a murine or a human fibroblast feeder layer. In one example, the feeder layer is mouse embryonic cell line (STO-1) or primary mouse embryonic fibroblasts, both treated with ultra-violet light or an anti-proliferative drug such as mitomycin C). Supportive adult human feeder cells are also exemplified by culture-expanded human bone marrow stromal cells (hMSCs) of passage 2 (p2) to p5, including hMSCs from multiple donors, which supported the growth of the H1 hES cell line under a serum-free condition (see published PCT Application No. WO04044158A2).

In one embodiment, the ES cells are cultured in the presence of FGF-2, a growth factor that prevents differentiation of ES cells. In one example, the cells are grown in the presence of about 1 to about 10 ng/ml of FGF-2. In several additional

examples, the medium includes about 3 to about 7 ng/ml of FGF-2, or about 5 ng/ml of FGF-2. In one embodiment, the ES cells are cultured for about 4 days to about 8 days. In another embodiment, the ES cells are cultured for about 5 days to about 7 days. The ES cells are cultured at temperature between about 35°C and about 40°C, or at about 37°C under an atmosphere which contains oxygen and between from about 1% to about 10%, or from about 1% to 5% CO_2, or at about 5% CO_2. In one embodiment, the media is changed about every 1 to 2 days (see U.S. Patent No. 5,670,372, herein incorporated by reference).

Generation of embryoid bodies (EB)

In one embodiment, embryoid bodies are generated from ES cells in suspension culture. Briefly, to form embryoid bodies, clusters of ES cells are disengaged from the tissue culture plates. Methods for disengaging cells from tissue culture plates are known and include the use of enzymes, such as trypsin or papain, and/or methyl ion chelators such as EDTA or EGTA, or commercially available preparations (e.g. see published PCT Application No. WO 00/27995).

The formation of embryoid bodies is well known in the art (see for example Conley et al., *Fetal Diagnosis and Therapy* 19:218-223, 2004; U.S. Patent No. 6,602,711). Generally, embryoid bodies are generated by re-plating expanded ES cells into dishes, such as bacterial dishes, where they do not form aggregates. Generally, the ES cells disengage from the tissue culture plates in clusters (e.g., aggregates of 10 or more ES cells, typically 50 or more cells). The clusters of ES cells are then dissociated to obtain a population of cells which includes a majority of (e.g., between about 50% and about 70%, or between about 75% and about 90%, or between about 80% and about 100%) individual cells. Methods for dissociating clusters of cells are likewise known. One method for dissociating cells includes mechanically separating the cells, for example, by repeatedly aspirating a cell culture with a pipette. In one embodiment, the ES cells are in an exponential growth phase at the time of dissociation to avoid spontaneous differentiation that tends to occurs in an overgrown culture.

The dissociated ES cells are then cultured in a medium, such as ES differentiation (or proliferation) medium, by re-plating at a density of about 1×10^5

to about 10 x 10^5 cells per milliliter (cells/ml), such as about 2 x 10^5 cells/ml. However, in contrast to the ES cell proliferation (in which the cells are grown on a tissue culture dish surface), embryoid bodies are generated in suspension, without the presence of a feeder layer. For example, to form embryoid bodies, the cells may be cultured on non-adherent bacterial culture dishes. In one embodiment, the cells are incubated from about 7 days to about 10 days, or about 7 to about 9 days, or for about 8 days. In one embodiment, the medium is changed every 1 to 2 days (see Martin et al., *Proc. Natl. Acad. Sci.*72:1441-1445, 1975; U.S. Patent No. 5,014,268, herein incorporated by reference).

In one embodiment, embryoid bodies are not generated, but a three dimensional matrix is utilized to form an artificial embryoid bodies. In this embodiment, a hollow fiber device or cell foam is used to create an artificial embryoid body. The cells are then cultured in the differentiation medium.

In one embodiment, the differentiation medium includes a differentiation factor, such as fibroblast growth factor-4 (FGF-4) at a concentration of about 50 ng/ml to about 1000 ng/ml, such as about 50 ng/ml to about 100 ng/ml fibroblast growth factor-4 (FGF-4), or a functional fragment thereof.

In another embodiment, the differentiation medium includes retinoic acid. In one specific, non-limiting example, the medium includes 1μM retinoic acid to about 10 μm retinoic acid, such as about 5μM retinoic acid.

In one specific, non-limiting example, differentiation medium or proliferation medium includes Dulbecco's Modified Eagle's Medium (DMEM), a serum replacement, L-glutamine, non-essential amino acids and beta-mercaptoethanol. Thus, an exemplary medium includes DMEM, 20% serum replacement, 1mm L-glutamine, 0.1 mM non-essential amino acids $\pm$ 0.1 mM b-mercaptoethanol. The ES differentiation medium contains FGF-2 as a mitogen, such as about 5ng/ml FGF-2.

Selection for neuronal precursors

The cells of the embryoid body (EB) are cultured to select for Central Nervous System (CNS) stem cells. To select for CNS stem cells, the EB cells are plated onto a surface that permits adhesion of CNS stem cells, for example an

extracellular matrix-coated surface. In specific examples, a laminin, or vitronectin coated surface is utilized. In one example, fibronectin is not utilized.

In one embodiment, the medium is a serum-free minimal essential medium, such as Dulbecco's Modified Eagles Medium (DMEM) or F12, or a combination of DMEM and F12. The serum-free medium is supplemented with nutrients. In one embodiment, the medium includes one or more of insulin, transferrin or selenium, or includes insulin, transferring and selenium. An example of a serum free media is ITSFn medium which includes DMEM and F12 in a ratio between 0.1:1 and 10:1 supplemented with between about 1 ng/ml to about 10 ng/ml insulin, about 20 nM to about 40 nM selenium chloride, about 40 ng/ml to about 60 ng/ml transferrin and without fibronectin.

In one embodiment, the medium includes fibroblast growth factor at a concentration of about 100 ng/ml to about 1000 ng/ml, such as about 50 ng/ml to about 100 ng/ml fibroblast growth factor-4 (FGF-4), FGF-2, or FGF-8, or a functional fragment of FGF-2, FGF-4, or FGF-8. In one specific example, the cells are cultured in the presence of FGF-4. In another embodiment, the medium includes retinoic acid. In one specific, non-limiting example, the medium includes 1 μM retinoic acid to about 10 μm retinoic acid, such as about 5 μM retinoic acid.

In one embodiment, the cells are incubated in the serum-free medium for between about 6 to about 30 days, such as about 6 to about 8 days, at a temperature between about 35°C and about 40°C. In another embodiment, the cells are incubated at 37°C under between about 1% and 10 % CO_2 atmosphere, or between about 5% and 10% CO_2 or under about 5% CO_2. In this embodiment, the medium is changed every 1 to 2 days.

At the end of the selection stage, the cell culture is enriched for neuronal precursor cells. Thus, in several specific non-limiting examples, the cell culture contains more than about 50% CNS stem cells, preferably more than about 80% CNS stem cells, more preferably more than about 90% CNS stem cells. The CNS stem cells can be identified by the cell-specific protein nestin. Additionally, transcriptional regulators typical of the cells in the midbrain and hindbrain, such as Pax2, Pax5, wnt1 and En1 are expressed by the CNS precursor cells.

Expansion of the CNS precursor cells

In one embodiment, the CNS precursor cells are expanded in a CNS expansion medium until the amount of cells increases about 10 fold. In another embodiment, the CNS precursor cells are expanded until the amount of cells increases from about 10 fold to about 100 fold. In one specific, non-limiting example, nestin positive cells are expanded in the presence of a growth factor. In another specific, non-limiting example, neuronal stem cells or precursor are expanded in the presence of a growth factor for about 6 to about 7 days.

A variety of culture media are known and are suitable for use in this step. Generally, the media includes a minimal essential media such as DMEM and/or F12, preferably a combination of DMEM and F12 (at a ratio between about 0.1:1 to 10:1). The medium can include one or more of insulin, transferrin, putrescine, selinite or progesterone. The medium can include insulin, transferrin, putrescine, selenite, and progesterone. In one example, the expansion culture medium includes N2 medium, or is entirely N2 medium. N2 medium is known in the art. In one specific example, the medium includes:

DMEM/F12 - Penicillin / Streptomycin;
BSA 100 mg/ml; Apo-transferrin 100 mg/ml; Putrescine 100 mM;
5 mg/ml Insulin; Progesterone 20 nM
sodium selenite 30 nM

The media can be supplemented with additional additives such as nutrients. Specific, non-limiting examples of these nutrients are shown in the table below

Additive	**Exemplary Concentration**
glucose	about 0.5 mg/ml to about 5.0 mg/ml
glutamine	about 0.01 mg/ml to about 0.1 mg/ml
sodium bicarbonate ($NaHCO_3$)	about 0.05 mg/ml to about 5.0 mg/ml
insulin	about 10 mg/ml to about 30 mg/ml
transferrin	about 50 mg/ml to about 150 mg/ml
putrescine	about 50 μM to about 150 μM
selenite	about 20 nM to about 40 nM
progesterone	about 10 nM to about 30 nM

In one specific, non-limiting example, the expansion medium includes between about 0.05 mg/ml and 5.0 mg/ml sodium bicarbonate, such as 1.0 mg/ml to 2.0 mg/ml sodium bicarbonate. Preferably the media does not include 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES).

The CNS expansion media may also be supplemented with neurologic agents to encourage differentiation into neuronal cells such as secreted signaling factors. In one specific, non-limiting example, the culture media includes between about 5 ng/ml to about 30 ng/ml basic fibroblast growth factor (bFGF, FGF-2) or epidermal growth factor (EGF), such as about 10 ng/ml to about 20 ng/ml bFGF (FGF-2). In one specific, non-limiting example, between about 10 ng/ml and about 20 ng/ml bFGF is included in the expansion media.

The CNS expansion media may also be supplemented with neurologic agents to increase the efficiency of the generation of midbrain dopaminergic neurons, such as factors that control dopaminergic and serotonergic cell fates during embryogenesis *in vivo*. In one embodiment, the media includes about 100 ng/ml to about 1000 ng/ml sonic hedgehog (Shh) protein (or functional fragments thereof), such as about between about 250 ng/ml and 500 ng/ml Shh, or about 25 ng/ml to about 200 ng/ml Shh. In another embodiment, the culture media includes about 50 ng/ml to about 100 ng/ml fibroblast growth factor-8 (FGF-8) (or functional fragments thereof). In one specific, non-limiting example, the media includes both FGF-8 and Shh.

Differentiation of the expanded CNS precursors

Differentiation of the expanded CNS precursors to form mature neuronal cells is induced by withdrawal of at least one neurologic agent, such as bFGF. The cells are cultured in the presence of medium, such as, but not limited to, N2 medium. The cells are cultured for about 5 to about 20 days, such as from about 7 to about 16 days, or from about 8 to about 15 days.

In one embodiment, treatment with FGF-4 during the generation of embryoid bodies (stage 2, see Fig. 8) and the selection of neuronal precursors (stage 3, see Fig. 8), followed by treatment with Shh and FGF-8 during the expansion of neuronal precursors (stage 4, see Fig. 8) leads to differentiation of human

dopaminergic neurons. In one example, at least 30%, such as at least 50%, of the neurons express tyrosine hydroxlyase. In another example, at least 90% of the neurons that express tyrosine hydroxylase also express the LIM homeodomain protein, Lmx1b. In yet another example, these cells are synaptically active.

In yet another embodiment, other biological active molecules are included in the media. These factors can include, but are limited to, factors to enhance dopaminergic neuron yield, such as between about 50 nM to about 500 nM ascorbic acid (AA). For example, about 100 nM to about 300 nM AA, or about 150 nM to about 250 nM AA is included. Without being bound by theory, ascorbic acid treatment tends to increase dopaminergic neuronal population in primary mesencephalic (midbrain) stem cell cultures. Typically, when ascorbic acid is added, greater than 40% and even greater than 45% of the neurons derived from ES cells express either dopamine or serotonin.

Optionally, the method also includes contacting the cells with other cells of the nervous system, such as astrocytes or glial cells. These cells can be from the same species, or from a different species (xenogeneic). In one embodiment, prior to or during use of the differentiated neuronal cells, the cells are contacted with supportive cells from the central nervous system, such as astrocytes or glial cells.

Simplified Method for Differentiating Neuronal Cells

Maintenance of Human ES Cells

Human ES cells are maintained using methods known in the art. In one specific example, the ES cells are maintained on a fibroblast feeder layer, such as, but not limited to, mouse embryonic fibroblasts prepared from a day 13.5 mouse fetus (e.g. a CF-1 mouse fetus). The production of feeder layers is well known in the art (see *Hum Reprod.*18(7):1404-9, 2003). In one example, a culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells.

The cells are maintained in a high glucose medium. One specific, non-limiting example, of a medium of use is DMEM High Glucose, which is commercially available. The medium is supplemented with a serum replacement,

such as with 10% to 30% v/v serum replacement, such as about 20% v/v serum replacement. One specific, non-limiting example of a serum replacement of use is Knockout Serum Replacement™, manufactured by Gibco.

Additive	**Range of Concentrations of Use**	**Exemplary Concentration of Use**
Glutamine	0.01 mg/ml to 0.1 mg/ml	1 mM
Nonessential Amino Acids*	0.01mM to 1.0 mM	0.1 mM
Beta-mercaptoethanol	0.01mM to 0,5 mM	0.1 mM
FGF-2	1ng/ml to 20 ng/ml	5 ng/ml

*Nonessential amino acids include

The ES cells are cultured at temperature between about 35°C and about 40°C, or at about 37°C under an atmosphere which contains oxygen and between from about 1% to about 10%, or from about 1% to 5% CO_2, or at about 5% CO_2. In one embodiment, the media is changed about every 1 to 2 days (see U.S. Patent No. 5,670,372, herein incorporated by reference).

Other methods and media for obtaining and culturing embryonic stem cells are known and are suitable for use (Evans et al., *Nature* 292:154-156, 1981; Martin et al., *Proc. Natl. Acad. Sci.* 78:7634-7636, 1981; Robertson et al., *Nature* 323:445-448, 1986; Doetschman et al., *Nature* 330:576-578, 1987; Thomas et al., *Cell* 51:503-512, 1987; Thomson et al., *Science* 282:1145-1147, 1998; and Shamblott et al., *Proc. Natl. Acad. Sci. USA* 95:13726-13731, 1998). The disclosures of these references are incorporated by reference herein.

The ES cells are then expanded *in vitro*. In one example, the human ES cells are plated in medium conditioned by embryonic fibroblasts. A "conditioned medium" can be prepared by culturing a first population of cells in a medium, and then harvesting the medium. The conditioned medium (along with anything secreted into the medium by the cells) may then be used to support the growth of a second population of cells. In one example, the conditioned medium is prepared by culturing mouse embryonic fibroblasts in a medium, and harvesting the medium.

Such media is well known in the art, and is commercially available (e.g. MEF-conditioned media available from R and D Systems). The cells are plated onto tissue culture plates coated with an extracellular matrix. The extracellular matrix can be laminin, or other suitable substrate, such as fibronectin, collagen, or an artificial extra-cellular matrix. During this expansion, the cells are grown in the absence of feeder cells, LIF, serum, or serum replacement. The medium, such as MEF condition media, is changed periodically. For example, the media is changed about every 3-5 days, such as about every four days.

Differentiation of Human ES Cells

In order to differentiate the human ES cells, the fibroblast-conditioned medium is replaced with a medium including one or more of insulin, transferrin or selenium, such as a medium including insulin, transferrin and selenium. In one example, the medium is ITS medium. ITSFn medium includes DMEM and F12 in a ratio between 0.1:1 and 10:1 supplemented with between about 1 ng/ml to about 10 ng/ml insulin, about 20 nM to about 40 nM selenium chloride, about 40 ng/ml to about 60 ng/ml transferrin and between about 1ng/ml to 10 ng/ml fibronectin. In one embodiment, the cells are incubated in this medium for between about 2 to about 20 days at a temperature between about 35°C and about 40°C. The media is changed about every 3-5 days, such as about every four days. In one example, the cells are incubated at 37°C under between about 1 % and 10 % CO_2 atmosphere, or between about 5% and 10% CO_2 or under about 5% CO_2.

Following growth in the medium including one or more of insulin, transferrin, and selenium, the cells are grown in a medium including one or more of insulin, transferrin, putrescine, selenite, and/or progesterone. In one example, the medium is N2 medium. This medium includes DMEM/F12 at a ratio of 1:1, and insulin, transferrin, progesterone, putrescine, and selenium, as described above.

Following growth in the medium including one or more of insulin, transferrin, putrescine, selenite, and/or progesterone, such as N2 medium, at least 30% of the cells are neuronal. In several examples, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% express Tuj1. In other examples, at least 20%, such as about 30%, 40%, 50%, 60% or more of the cells are dopaminergic.

For example, growth in the N2 medium, at least 20%, such as at least about 30%, at least about 40%, at least about 50%, at least about 60% or more of the cells express tyrosine hydroxylase. At least some of the differentiated neurons in the cell culture are synaptically active. Typically, at least about 70%, about 80% or about 90% such as at least about 95% of the cells in the culture are synaptically active differentiated neurons (e.g., dopaminergic and/or serotonergic neurons).

The ES cells or the differentiated cells can be transfected with a gene of interest. Optionally, the method also includes contacting the cells with other cells of the nervous system, such as astrocytes or glial cells. These cells can be from the same species, or from a different species (xenogeneic). In one embodiment, prior to or during use of the differentiated neuronal cells, the cells are contacted with supportive cells from the central nervous system, such as astrocytes or glial cells.

Differentiation of Mesodermal Cells such as Hepatocytes

A method is disclosed herein for differentiating ES cells into hepatocytes. In one embodiment, the method includes expanding primate ES cells, such as human ES cells (see above for a complete description of the expansion of ES cells). However, the expansion of human ES cells prior to differentiation is not required to perform the method disclosed herein. However, to increase the number of hepatocytes cells formed, ES cells can be expanded prior to embryoid body formation. In general, undifferentiated embryonic stem (ES) cells are cultured in ES proliferation media to expand the number of cells, as described above.

Following expansion, embryoid bodies are produced. In one embodiment, embryoid bodies are generated from ES cells in suspension culture. Briefly, to form embryoid bodies, clusters of ES cells are disengaged from the tissue culture plates. Methods for disengaging cells from tissue culture plates are known and include the use of enzymes, such as trypsin or papain, and/or methyl ion chelators such as EDTA or EGTA, or commercially available preparations (e.g. see published PCT Application No. WO 00/27995). The formation of embryoid bodies is well known in the art (see for example Conley et al., *Fetal Diagnosis and Therapy* 2004;19:218-223; U.S. Patent No. 6,602,711), and is described in detail above.

The dissociated ES cells are cultured in a medium, such as ES differentiation (or proliferation) medium, by re-plating at a density of about 1 x 10^5 to about 10 x 10^5 cells per milliliter (cells/ml), such as about 2 x 10^5 cells/ml. However, in contrast to the ES cell proliferation (in which the cells are grown on a tissue culture dish surface), embryoid bodies are generated in suspension, without the presence of a feeder layer. For example, to form embryoid bodies, the cells can be cultured on non-adherent bacterial culture dishes. In one embodiment, the cells are incubated from about 7 days to about 10 days, or about 7 to about 9 days, or for about 8 days. In one embodiment, the medium is changed every 1 to 2 days (see Martin et al., *Proc. Natl. Acad. Sci.*72:1441-1445, 1975; U.S. Patent No. 5,014,268, herein incorporated by reference).

In order to generate hepatocytes, embryoid bodies can be treated with a factor that activates the wnt signaling pathway. The wnt family of secreted signaling molecules play a role in embryonic induction, cell polarity generation, and cell fate specification. The wnt pathway involves activation of β-catenin-dependent transcription and is evolutionarily conserved from *C. elegans* to humans. Wnt binds to two co-receptors, the frizzled type seven-transmembrane domain receptor and the low density receptor-related protein. These interactions cause β-cantenin stabilization (see Liu et al., *Mol. Cell. Biol.* 23: 5825-5835, 2003).

In one embodiment, embryoid bodies are contacted with an effective amount of a factor that activates the wnt signaling pathway, such as an effective amount of lithium or a salt thereof. In one example, embryoid bodies are contacted with LiCl, such as about 1 to about 100 mM LiCl, such as about 1 mM LiCl to about 20 mM LiCl, or about 10mM LiCl in medium. In another example, primate embryoid bodies are contacted with 1-20 mM LiCl from day two to day four of embryoid body formation. Without being bound by theory, lithium activates the wnt signaling pathway.

In another embodiment, embryoid bodies are contacted with an effective amount of serum, such as fetal bovine serum. In one example, embryoid bodies are contacted with 1% to 20% serum, such as 5% to 15% serum, or about 10% serum. The hepatic cells are distinguished using any means known to one of skill in the art. In additional embodiments, the expression of markers of fetal hepatoblasts (such as

GATA4, hepatocyte nuclear factor -3 β (HNF3β), OV6, CK8, Ck18 and/or albumin) is assessed. The OV6 antibody recognizes cytokeratins 14 and 19, and cytokeratin 8 expression. More differentiated hepatocytes express cytokeratin 18, HNF3β, and albumin. The cells can also be evaluated to demonstrate that they express gamma-glutamyl-transpeptidase (GGT). In several examples, the differentiated cells include at least about 30% hepatocytes, such as at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% hepatocytes.

Exemplary Use of the Differentiated Cells

The present methods can be employed to produce neuronal cells in order to deliver the cells, or molecules expressed by these cells, to the brain for diagnosis, treatment or prevention of disorders or diseases of the CNS, brain, and/or spinal cord. These disorders can be neurologic or psychiatric disorders. These disorders or diseases include brain diseases such as Alzheimer's disease, Parkinson's disease, Lewy body dementia, multiple sclerosis, epilepsy, cerebellar ataxia, progressive supranuclear palsy, amyotrophic lateral sclerosis, affective disorders, anxiety disorders, obsessive compulsive disorders, personality disorders, attention deficit disorder, attention deficit hyperactivity disorder, Tourette Syndrome, Tay Sachs, Nieman Pick, and other lipid storage and genetic brain diseases and/or schizophrenia. The method can also be employed in subjects suffering from or at risk for nerve damage from cerebrovascular disorders such as stroke in the brain or spinal cord, from CNS infections including meningitis and HIV, from tumors of the brain and spinal cord, or from a prior disease. The method can also be employed to deliver agents to counter CNS disorders resulting from ordinary aging (e.g., insomnia or loss of the general chemical sense), brain injury, or spinal cord injury.

The present method can be employed to deliver agents to the brain for diagnosis, treatment or prevention of neurodegenerative disorders. Sublingual, conjunctival or transdermal administration of an agent to peripheral nerve cells of the trigeminal and other sensory neural pathways innervating the skin or the conjunctival or oral mucosa, purported entryway for causative agents of brain diseases, can help protect against disease in these nerve cells and regenerate injured

nerve cells, thereby forestalling the subsequent spread of disease to susceptible areas of the CNS, brain, and/or spinal cord.

The application of cells can also be used to replace or augment peripheral cells and neurons that are injured by neurotoxins and other insults. This method of treatment is beneficial in cases of Alzheimer's disease where an environmental factor is suspected of being one of the causative agents of the disease. Application of cells can also be used to affect the loss of smell or of the general chemical sense which may be associated with neurodegenerative diseases and ordinary aging.

The cells can also be used in the treatment of Parkinson's disease. The principal therapeutic target in the brain for Parkinson's disease is the substantia nigra which extends forward over the dorsal surface of the basis peduncle from the rostral border of the pons toward the subthalamic nucleus. Other therapeutic target areas are the locus ceruleus which is located in the rostral pons region and the ventral tegmental area which is located dorsomedial to the substantia nigra.

After the differentiated neuronal cells are formed according to a cell culturing method described above, the cells are suspended in a physiologically compatible carrier. The carrier can be any carrier compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. Those of skill in the art are familiar with physiologically compatible carriers. Examples of suitable carriers include cell culture medium (e.g., Eagle's minimal essential media), phosphate buffered saline, and Hank's balanced salt solution +/- glucose (HBSS). In one embodiment, supporting cells, such as glia or astrocytes, can be added. These cells can be from the same species as the neuronal cells, or from a different species. Thus, in one embodiment, human ES cells are differentiated to human neuronal cells, and administered to the subject in conjunction with human glia or astrocytes. In another embodiment, the human neuronal cells are administered with murine astrocytes or glial cells to the subject.

The volume of cell suspension administered to a subject will vary depending on the site of implantation, treatment goal and amount of cells in solution. Typically the amount of cells administered to a subject will be a therapeutically effective amount. For example, where the treatment is for Parkinson's disease, transplantation of a therapeutically effective amount of cells will typically produce a reduction in

the amount and/or severity of the symptoms associated with that disorder, e.g., rigidity, akinesia and gait disorder.

In one example, a severe Parkinson's patient needs at least about 100,000 surviving dopamine cells per grafted site to have a substantial beneficial effect from the transplantation. As cell survival is low in brain tissue transplantation in general (5–10%) at least 1 million cells are administered, such as from about 1 million to about 4 million dopaminergic neurons are transplanted.

In one embodiment, the cells are administered to the subject's brain. The cells may be implanted within the parenchyma of the brain, in the space containing cerebrospinal fluids, such as the sub-arachnoid space or ventricles, or extaneurally. Thus, in one example, the cells are transplanted to regions of the subject which are not within the central nervous system or peripheral nervous system, such as the celiac ganglion or sciatic nerve. In another embodiment, the cells are transplanted into the central nervous system, which includes all structures within the dura mater.

Typically, the neuronal cells are administered by injection into the brain of the subject. Injections can generally be made with a sterilized syringe having an 18-21 gauge needle. Although the exact size needle will depend on the species being treated, the needle should not be bigger than 1 mm diameter in any species. Those of skill in the art are familiar with techniques for administering cells to the brain of a subject.

It should be noted that cells produced by the methods disclosed herein can also be used in to screen pharmaceutical agents to select for agents that affect specific human cell types, such as agents that affect neuronal cells.

Screening for Agents that Affect the Differentiation of Human ES Cells

A method is provided herein for selecting an agent that affects the differentiation of human ES cells. In one embodiment, the agent affects the differentiation of human ES cells into a differentiated cell fate. In one embodiment, the agent affects the differentiation of ES cells into neurons. The neurons can be dopaminergic neurons, but can also be adrenergic, serotinergic, or motor neurons. In another embodiment, the agent affects differentiation into glial cells. In another

embodiment, the agent affects differentiation into cells of an endodermal lineage, such as hepatocytes.

The test compound can be any compound of interest, including chemical compounds, small molecules, polypeptides or other biological agents (for example antibodies or cytokines). In several examples, a panel of potential agents are screened, such as a panel of cytokines or growth factors is screened.

Methods for preparing a combinatorial library of molecules that can be tested for a desired activity are well known in the art and include, for example, methods of making a phage display library of peptides, which can be constrained peptides (see, for example, U.S. Patent No. 5,622,699; U.S. Patent No. 5,206,347; Scott and Smith, *Science* 249:386-390, 1992; Markland et al., *Gene* 109:13 -19, 1991), a peptide library (U.S. Patent No. 5,264,563); a peptidomimetic library (Blondelle et al., *Trends Anal Chem.* 14:83-92, 1995); a nucleic acid library (O'Connell et al., *Proc. Natl Acad. Sci., USA* 93:5883-5887, 1996; Tuerk and Gold, *Science* 249:505-510, 1990; Gold et al., *Ann. Rev. Biochem.* 64:763-797, 1995); an oligosaccharide library (York et al., *Carb. Res.* 285:99-128, 1996; Liang et al., *Science* 274:1520-1522, 1996; Ding et al., *Adv. Expt. Med. Biol.* 376:261-269, 1995); a lipoprotein library (de Kruif et al., *FEBS Lett.* 3 99:23 2-23 6, 1996); a glycoprotein or glycolipid library (Karaoglu et al., *J Cell Biol.* 130.567-577, 1995); or a chemical library containing, for example, drugs or other pharmaceutical agents (Gordon et al., *J Med. Chem.* 37.1385-1401, 1994; Ecker and Crooke, *BioTechnology* 13:351-360, 1995). Polynucleotides can be particularly useful as agents that can alter a function of ES cells because nucleic acid molecules having binding specificity for cellular targets, including cellular polypeptides, exist naturally, and because synthetic molecules having such specificity can be readily prepared and identified (see, for example, U.S. Patent No. 5,750,342).

In one embodiment, for a high throughput format, ES cells can be introduced into wells of a multiwell plate or of a glass slide or microchip, and can be contacted with the test agent. Generally, the cells are organized in an array, particularly an addressable array, such that robotics conveniently can be used for manipulating the cells and solutions and for monitoring the ES cells, particularly with respect to the function being examined. An advantage of using a high throughput format is that a

number of test agents can be examined in parallel, and, if desired, control reactions also can be run under identical conditions as the test conditions. As such, the methods disclosed herein provide a means to screen one, a few, or a large number of test agents in order to identify an agent that can alter a function of ES cells, for example, an agent that induces the ES cells to differentiate into a desired cell type, or that prevents spontaneous differentiation, for example, by maintaining a high level of expression of regulatory molecules

The cells are contacted with test compounds sufficient for the compound to interact with the cell. When the compound binds a discrete receptor, the cells are contacted for a sufficient time for the agent to bind its receptor. In some embodiments, the cells are incubated with the test compound for an amount of time sufficient to affect phosphorylation of a substrate. In some embodiments, cells are treated *in vitro* with test compounds at 37°C in a 5% CO_2 humidified atmosphere. Following treatment with test compounds, cells are washed with Ca^{2}+ and Mg^{2}+ free PBS and total protein is extracted as described (Haldar et al., *Cell Death Diff.* 1:109-115, 1994; Haldar et al., *Nature* 342:195-198, 1989; Haldar et al., *Cancer Res.* 54:2095-2097, 1994). In additional embodiments, serial dilutions of test compound are used.

In one embodiment, embryoid bodies (EBs) can be contacted with the agent. When the effect on the differentiation of neuronal cells is being assessed, the cells can be contacted with the agent at the same time as FGF-4, or at the same time as FGF-2, FGF-8 and Shh. Following differentiation, the number of differentiated cells present in the culture is compared to identical cells that were not exposed to the test compound. Suitable methods are disclosed herein to assess the differentiated state of a cell. For example, the expression of TH and/or albumin can be assessed (see the examples section below).

The disclosure is illustrated by the following non-limiting Examples.

EXAMPLES

The vertebrate embryo contains three tissues, ectoderm, endoderm and mesoderm, which are supported by cells that do not contribute to the embryo but play critical roles (the extra-embryonic ectoderm and endoderm). In normal development these cell types emerge in a series of steps, gastrulation, where the highly ordered organ structure of the vertebrate fetus emerges. ES cells are regarded as equivalent to the inner cell mass, cells of the blastula that give rise to the major cell types of the body through the process of gastrulation. However, when ES cells differentiate *in vitro*, the exquisite order of the early embryo is absent. Very little is known about the differentiation of cell types in the absence of the normal organization of the embryo.

The great interest in the clinical value of human ES cells currently rests predominantly on previous data generated with mouse ES cells. Of the many problems still to be resolved in the quest to make clinical use of human ES cells, perhaps the most fundamental is to isolate a specific cell type of clinical value in high yield. The importance of generating data with human ES cells is emphasized by the clear differences in antigen expression between undifferentiated mouse and human ES cells. In addition, mouse ES cells are expanded and maintained in an undifferentiated state by the cytokine, LIF while human ES cells are unresponsive to LIF. FGF-2 (also known as bFGF) is used to expand undifferentiated human ES cells and, in contrast, drugs that inhibit FGF signaling promote the expansion of undifferentiated mouse ES cells. Thus, the proliferation of human and mouse ES cells likely is controlled by distinct molecular mechanisms.

Transplantation into the blastocyst stage of embryonic development shows that mouse embryonic stem (ES) cells are pluripotent, capable of differentiating into all of the adult lineages including the germ line. For ethical reasons, the pluripotency of human ES cells cannot be defined in this way, but human ES cells do form teratomas containing many different cell types. Additional studies *in vitro* demonstrate that altering conditions can be used to differentiate ES cells into several different cell types. However, the range of cell types and the efficiency of differentiation are still not known. Disclosed herein are *in vitro* conditions that can

be used to generate several major cell types of the embryo. These results demonstrate that specific terminal cell fates can be readily derived from human ES cells.

As disclosed herein, human ES cells can rapidly form embryoid bodies (EBs), cell aggregates that contain the major embryonic and extra-embryonic cell types. The proportion of early cell types can be regulated by application of simple external signals giving EBs that are highly enriched in specific cell states. In addition, it is demonstrated that dopaminergic neurons can be efficiently derived from EBs containing large numbers of neural precursor cells. These results show that the proliferative mechanisms are different in mouse and human ES cells. Like the mouse system, human ES cells respond to physiologically appropriate developmental signals as they acquire distinct neuronal fates, although these signals are unique. The signals can be used to develop techniques to obtain specific functional cell types, a necessity for rapid and general clinical application, as demonstrated in the experimental results set forth below.

Example 1

Confirmation of the Undifferentiated State of Human ES Cells

Several human ES cell lines are available for study under the NIH guidelines. Little is known about the differentiation properties of these cells but there are reports showing that differentiated fates have been obtained (see He et al., *Circ Res.* 93(1):32-9, 2003; Epub 2003 Jun 05; Lavon and Benvenisty, *Trends Cardiovasc Med.* (2):47-52, 2003; Zhang et al., *Nat Biotechnol.* 19(12):1129-33, 2001; Carpenter et al., *Exp Neurol.* 172(2):383-97, 2001; Schuldiner et al., *Brain Res.* 913(2):201-5, 2001; Kehat et al., *J Clin Invest.* 8(3):407-14, 2001; Schuldiner et al., *Proc Natl Acad Sci U S A* 97(21):11307-12, 2001).

However, in many of these studies, the efficiency and mechanisms of differentiation are not known, and a protocol to differentiate cells into a particular fate is not disclosed.

The differentiation of a human ES cell line (HSF-6, NIH code# UC06, passage 38-102) was controlled to obtain differentiated cells through defined precursors. The starting cells are undifferentiated. HSF6 cells were grown in the

presence of FGF-2 (5 ng/ml) on mouse embryonic fibroblasts. The undifferentiated ES cells expressed the transcription factor Oct-4, the surface antigen SSEA-4 and alkaline phosphatase. These markers characterize the undifferentiated state (Figes et al., *Curr Biol.* 11(7):514-8, 2001). In the mouse, Oct4 regulates an early pluripotent state and is rapidly down regulated, generating the cells of the inner cell mass and the trophectoderm (Nichol et al., *Cell* 95(3):379-91, 1998). Almost all the cells express high levels of Oct4. Karyotypic analysis of the cells at early and late passage showed that the cells contained a normal complement of chromosomes.

Analyses of digital images showed the regulation of endodermal fate in human ES-derived embryoid bodies (EBs) by ectopic LiCl/beta-catenin signaling. Undifferentiated human HSF-6 cells expressed Oct-4 and SSEA-4, markers of pluripotent human ES cells. In some cases, a thin layer of GATA4+AFP+ cells was found at the surface of control embryoid bodies. A few GATA-4+AFP- cells were observed within these EBs. In all of the embryoid bodies treated with LiCl, there was a thick GATA-4+AFP+ surface layer of endoderm and numerous GATA-4+AFP- cells are contained beneath this layer. In serial sections, nuclear localization of beta-catenin is observed in AFP+ regions, but not in AFP- regions within LiCl-treated EBs.

These observations suggest that the HSF-6 cells are pluripotent, undifferentiated cells.

Example 2

Developmental Potential of HSF6 Cells

The developmental potential of HSF6 cells was then determined *in vitro*. The strategy used to differentiate ES cells was to first allow the cells to form aggregates (embryoid bodies, EBs) that in the mouse are known to contain the early cell types with precise kinetics (Fehling et al., *Development* 130(17):4217-4227, 2003). Embryoid bodies are generated by re-plating ES cells at a specific density (2 x 10^5 /ml) into bacterial culture dishes where they do not attach to the surface and instead form aggregates. At first these aggregates, simple EBs, contain two cell types. The inner cells represent the somatic cells of the embryo and the outer cells

are extra-embryonic cells that form the supporting tissues particularly required in mammalian development (Abe et al., *Exp Cell Res.* 229(1):27-34, 1996).

As human ES cells develop more slowly than mouse ES cells, the duration of this initial step was extended to 8 days. At this point, a few aggregates showed the typical morphology of 'simple' EBs. Alpha-fetoprotein (AFP) and GATA4 are expressed in visceral endoderm (Belinska and Wilson, *Mech Dev.* 65(1-2):43-54, 1997) and cells expressing these proteins can be found at the surface of these 8-day aggregates. Similar to the mouse, beyond 8 days in culture many EBs contained a cavity or "cyst." Without being bound by theory, this data indicates that there is no evidence of primitive endodermal fates in the human EBs.

The wnt signal pathway influences the function of the visceral endoderm at early stages of vertebrate embryogenesis (Huelsken et al., *J Cell Biol.* 148(3):567-78, 2000). Lithium chloride is known to activate the wnt signaling pathway and this activation can be monitored by the nuclear localization of β-catenin (Giles and Clevers, *Biochim Biophys Acta.* 1653(1):1-24, 2003). When EBs were treated with 10mM LiCl from day 2-4 and analyzed at day 8 of cell aggregation there was a marked increase in the number of cells in the peripheral location that expressed AFP and GATA4. LiCl treatment also caused an increase in AFP- and GATA4+ cells in the interior of the EBs. It should be noted that LiCl can be used at a concentration of about 1-500mM, such as about 5 to about 100 mM, or about 10 to about 50 mM.

Immunohistochemistry was used to show activated nuclear β-catenin in peripheral regions with AFP and GATA4 co-expression. These results show that LiCl activates the wnt pathway and that human ES cells can form EBs containing extra-embryonic cells of the primitive endoderm. These results show that extra-embryonic cell differentiation can be manipulated by simple external signals. Thus, the embryonic cell types present in the EBs can be manipulated.

Example 3

Manipulation of ES Cells to Form Derivatives of the Mesoderm, Ectoderm, and Endoderm

The major cell types of the embryo form at gastrulation, these events occur between 2 and 3 weeks of human development. Many of the processes of gastrulation are fundamental to animals with a three-layered (tri-laminar) organization, an outer layer of ectoderm, an inner endoderm and an intermediate layer of mesoderm. It was determined that three basic cell types, as well as germ cells, could be formed from EBs.

The LiCl treated EBs show increased numbers of GATA4+, AFP- cells. This pattern of gene expression is consistent with differentiation to embryonic endoderm or cardiac mesoderm (Crispino et al., *Genes Dev.* 15(7):839-44, 2001; Rossi et al., *Genes Dev.* 15(15):1998-2000, 2001). Experiments in Xenopus and in the mouse suggest that mesoderm and neurectoderm are alternate fates of an early cell (Bell et al., *Development* 130(7):1381-9, 2003). The brachyury gene, first identified in the mouse, is thought to play a critical role in the differentiation of the mesoderm during gastrulation in all species that have a trilaminar organization (Ciruna and Rossant, *Dev Cell.* 1(1):37-49, 2001). Early neurectodermal cells express the transcription factor Sox1 (Pevny et al., *Development* 125(10):1967-78, 1998) and the intermediate filament protein nestin (Lendahl 1990). An antibody against Brachyury recognizes a nuclear protein in some EB (see Figs 1A-B). The Brachyury positive cells can vary. In contrast, many EBs express Sox1 and nestin. These results suggest that endodermal, mesodermal and ectodermal fates are generated in the EBs. It was noted that germ cells could also be generated from EBs.

Example 4

Differentiation of EBs

It has been shown that differentiated cells can migrate out of mouse EBs and give rise to CNS cell types with high efficiency (Okabe et al., *Mech Dev.* 59(1):89-102, 1996; Lee et al., *Nat Biotechnol.* 18(6):675-9, 2000). Day 8 EBs treated with LiCl between days 2 and 4 were re-plated into dishes that support cell attachment.

Most EBs attach and many polygonal cells migrated out of the EBs over the ensuing two days. Many cells react with the OV6 antibody that recognizes cytokeratins 14 and 19 (Fig. 2A). This population contains many cells that co-express GATA4 and cytokeratin 8 (Fig. 2B). These markers are commonly found in hepatic precursors. Differentiation for a further 3 days generates cells that express albumin and co-express albumin and AFP (Fig 2C). The albumin positive cells also co-express cytokeratin 18 (Fig. 2D). These data suggest that LiCl treated EBs can generate endodermal precursors that differentiate into hepatocytes.

When mouse EBs are plated onto fibronectin coated dishes, nestin-positive cells dominate that migrate across the surface. These cells proliferate in response to FGF-2 and differentiate into neurons, astrocytes and oligodendrocytes when the mitogen is withdrawn (Lee et al, *Nat Biotechnol.* 18(6):675-9, 2000; Brustle et al., *Science* 285(5428):754-6, 1999). This protocol contains five differentiation steps and can very efficiently generate dopaminergic neurons that share many developmental characteristics and mature functions with dopamine neurons found in the midbrain (Kim et al., *Nature* 418(6893):50-6, 2002). These data provide some of the strongest evidence that a specific functional cell can be derived *in vitro* from mouse ES cells and can function when replaced into a disease model. In a related experiment, mouse ES cells were shown to generate spinal cord motor neurons (Wichterle et al., *Cell* 110(3):385-97, 2002). In addition, a variation of the protocol has been used to generate neurons and glia from human ES cells that survive *in vivo*. But this study utilized only murine cells, and the mechanisms and factors required to efficiently differentiate specific CNS cell types *in vitro* was not analyzed in this study. Thus, the production of *human* neurons in response to developmentally appropriate signals was assessed.

EBs (Stage 2) generated under control conditions were transferred to tissue culture plates and cells did migrate onto the fibronectin coated surface. However, these cells had morphologies that were quite distinct from neural precursors (Fig. 4). The effects of FGF-2, FGF-4, and FGF-8 at stages 2 and 3 on ES cell neurogenesis were examined.

Immunocytochemical analysis at stage 5 revealed that all three FGFs increased neural specification. In particular, FGF-4 profoundly promoted neural

differentiation. In the presence of FGF-4, brachyury expression was diminished and neural precursors were more abundant in the EBs. FGF-4 increased the number of Sox1-positive cells from 45.31% ± 7.08% to 74.59% ± 3.91%. The distribution of the Sox1 and nestin positive cells in the EBs was also altered by exposure to FGF-4. Importantly, the number and size of the treated EBs was significantly larger than controls (Fig. 1B). In FGF-4 treated cultures, the majority of cells migrating from the EBs at stage 3 have the morphology of neural progenitors with less cell death than with mouse ES cells (Fig. 3C-3D).

For stage 4, the cells were transferred to poly-L-ornithine-coated plates in N2 medium containing FGF-2 (20 ng/ml) for 8 days. Under these conditions, a morphologically homogenous population of nestin-positive cells proliferated readily (Figs. 3E-3F). In contrast to mouse cells at stage 4, poly-L-ornithine in the absence of fibronectin is highly selective for nestin-positive human ES-derived neural precursors. An important feature of this strategy is that removal of the mitogen in the final step, stage 5, triggers a synchronous differentiation of the precursor cells. When human cells were taken through the 5-step protocol 60.9% ± 5.9% of the cells expressed the neuronal specific tubulin TuJ1 and adopted neuronal morphologies (Figs. 3G-3H). $GFAP^+$ or $O4^+$ astrocytes and oligodendrocytes were also seen (<1%).

Example 5

Effect of Factors on Neuronal Cell Differentiation

Several factors have been proposed to influence both the number and type of neurons that are generated from mouse ES cells. In addition to FGF-4, during stages 2 and 3 Brain Derived Neurotrophic Factor (BDNF), retinoic acid (RA) and noggin were tested (see Chao, *Nat Rev Neurosci.* 4(4):299-309, 2003). BDNF and noggin did not promote the expansion of neuronal precursors. In contrast, addition of retinoic acid (RA, 5 μM) in stages 2 and 3 increased the population of neuronal cells at stage 5. RA and FGF-4 promote neurogenesis to a similar extent but FGF-2 and 8 are significantly less effective. These results show that stage 4 cells derived from human ES cells are highly enriched for neuronal precursors (see Fig. 3).

In the mouse ES cell system, RA increases the number of cells expressing the spinal cord specific gene Hoxc6, at the expense of Otx2+ and En1+ cells that are found in fore- and mid-brain precursors (Wichterle et al., *Cell* 110(3):385-97, 2002). The transcription factor HB9 is expressed in the motor neuron lineage *in vivo* and RA treated mouse EB cells *in vitro* (Arber et al., *Neuron.* 23(4):659-74, 1999). RA treatment of differentiating human ES cells increased the number of HB9+ precursors at stage 2 and neurons at stage 5. No HB9 positive cells were seen at stage 2 or later without RA treatment (Fig. 3). This result suggests that RA control the anterior-posterior identity of CNS precursors at stage 2.

Dorsoventral patterning of the central nervous system is established by antagonizing gradients of the diffusible morphogens, sonic hedgehog factor (Shh) and Bone Morphogenetic Protein (BMP). Shh, from the floor plate, and FGF-8 produced by the isthmic organizer specify dopamine neurons during midbrain development (Ye et al, *Cell* 93(5):755-66, 1998). These factors can promote ventral midbrain dopaminergic neuron fate in mouse ES cell cultures. Following exposure to FGF-4 in stages 2 and 3, many human nestin$^+$ cells at stage 4 express the transcription factor engrailed-1 found in the mouse caudal midbrain and rostral hindbrain (Fig. 4). Treatment with Shh/FGF-8 during the expansion of nestin+ precursor cells (stage 4) increased the percentage of tyrosine hydroxylase (TH)+ cells up to 58% of the neurons (Figs. 6E and 6G; 57.7 ± 9.8%), compared with control (23.1± 3.8%). The number of HB9+ neurons was also significantly increased by Shh treatment at stage 4.

Although neural differentiation is significantly enhanced by retinoic acid, the posteriorizing effect of RA was also seen in a clear reduction of TH-positive midbrain dopamine neurons. Midbrain specific markers were used to further define the properties of these TH+ cells. The LIM homeodomain protein, Lmx1b, is necessary for the induction of the homeodomain gene Ptx3 in midbrain dopamine neurons and is also necessary for the survival of these cells (Smidt et al., *Proc Natl Acad Sci USA* 94(24):13305-10, 1997; Simon et al., *Ann N Y Acad Sci.* 991:36-47, 2003). At stage 5, over 90% of the TH$^+$ neurons express Lmx1b suggesting that the TH$^+$ neurons are midbrain dopamine neurons (Fig. 4). These results suggest that the dorso-ventral and rostro-caudal identity of ES cell-derived human CNS

precursors can be controlled by physiologically appropriate signals to generate specific neurons types.

It is important to establish if these cells can function as neurons. To monitor presynaptic functions in hES derived neurons, styryl dye FM4-64 was used to label vesicles in an activity-dependent manner and to image vesicle recycling at the level of individual synaptic terminals (Cochilla et al., *Annu Rev Neurosci.* 22:1-10, 1999). Neurons were differentiated in stage 5 in the presence of mouse astrocytes to support functional synapse formation (Blondel et al., *J Neurosci.* 20(21):8012-20, 2000). These neurons developed elaborate morphologies and antibodies against presynaptic proteins identified numerous positive structures with the dimensions of presynaptic terminals (Fig. 5). A characteristic punctate pattern along neuronal processes could also be detected by means of FM4-64 imaging (Fig. 5C). When a buffer containing 50 mM KCl was applied, a fraction (~25%) of these FM4-64 positive puncta released dye with accelerated kinetics (Fig. 5E).

The capacity of the TH+ neurons to synthesize and release dopamine when depolarized was assessed by reverse phase HPLC. The concentrations of L-DOPA, DA and the DA metabolite, DOPAC in the non-treated cultures were 274.9 ± 22.8 pmol/ml, 4.98 ± 0.5 pmol/ml and 4.13 ± 0.67 pmol/ml (mean ± S.E.M.), respectively. When the cultures were treated with Shh and FGF-8 in stage 4 the values increased significantly ($p<0.01$) up to 481.2 ± 22.8 pmol/ml, 17.74 ± 1.2 pmol/ml and 27.14 ± 2.62 pmol/ml (Fig. 5). The presence of nor-adrenaline and adrenaline was not observed in these cultures using an assay with a detection limit of 70 pg/ml and 120 pg/ml, respectively. In response to a depolarizing stimulus (56 mM K^+), ES cell–derived neurons released dopamine into the culture medium (Fig. 5). These results suggest the neurons synthesize and secrete dopamine through voltage dependent mechanisms.

It should be noted that these experiments demonstrate that human ES cells can be differentiated into electrically mature neurons that have the ability to fire sustained bursts of action potentials and exhibit synaptic vesicle release.

Example 6

Methods for Examples 7-10

Cell Culture: Human embryonic stem cells (HSF-6 line, available from the University of California, San Francisco) were cultured on mouse embryonic fibroblasts (MEF) according to the protocol, provided by the University of California, San Francisco in human ES culture medium [DMEM (Invitrogen, without sodium pyruvate) supplemented with 20% KnockOut™ Serum Replacement (Invitrogen), 1 mM glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma)] supplemented with 5 ng/ml FGF2 (R&D systems).

All stages of the differentiation protocol are according to the above-described five stage protocol for the differentiation of mouse ES cells into dopaminergic neurons, adapted to human ES cells (Fig. 3). To induce formation of embryoid bodies (EBs) (stage 2), hES colonies were harvested after treatment with 1.5 mg/ml collagenase type IV (Invitrogen), separated from the MEF feeder cells by gravity, gently triturated to break up colonies into small aggregates, resuspended in human ES culture media and transferred to non-adherent suspension culture dishes (Corning). Unless otherwise noted, human ES cell aggregates were cultured for 8 days in ES culture media (change of media every other day) supplemented with various factors [50 ng/ml FGF2, 50 ng/ml FGF4, 50 ng/ml FGF8, 50 ng/ml BDNF, .2 μM retinoic acid, 50 ng/ml BMP4 (all R&D systems); 10 % fetal bovine serum (Invitrogen), or 10 mM LiCl (only between day 2 and day 4; Sigma)].

To induce differentiation into endodermal cell fates, human EBs after LiCl or serum treatment were plated onto adherent tissue culture dishes in either ITSFn Medium supplemented with 5 μg/ml fibronectin, or 10% FBS in DMEM for up to 8 days.

To induce differentiation into neuroectodermal cell fates, human EBs were plated onto adherent tissue culture dishes in ITSFn Medium supplemented with 5 μg/ml fibronectin and indicated growth factors (stage 3). After 8 days of selection of nestin positive cells in stage 3, cells were dissociated using 0.05% trypsin/0.04% EDTA solution (Invitrogen) and plated on poly-L-ornithine (15 μg/ml, Sigma)-

coated tissue culture plates at a density of 2 x 10^5 cells/cm^2 in N2 medium containing FGF-2 (20 ng/ml, R&D systems) in the presence or absence of murine N-terminal fragment of Shh (500 ng/ml, R&D systems) and murine FGF-8 isoform b (100 ng/ml, R&D systems). After 6-8 days of expansion of nestin positive cells in stage 4, medium was replaced by N2 medium without FGF-2, FGF-8 and Shh to terminally differentiate into neurons for 8 to 15 days (stage 5a). For *in vitro* maturation experiments (stage 5b), cells were harvested as cell clusters (Stage 5, day 8) using HBSS and transferred onto confluent glial beds. Glial beds were prepared from E16.5 mice on 12mm glass coverslips treated with 3 mg/ml Vitrogen™ (Cohesion, Palo Alto, CA) and 1 mg/ml Poly-D-Lysine.

Immunostaining: Cells were fixed in 4% paraformaldehyde in PBS. EBs were fixed in 4% paraformaldehyde, transferred to 20% sucrose overnight, frozen in O.C.T compound (Tissue Tek®, Sakra) and sectioned at 10 μm. Immunostaining was carried out using standard protocols. The following antibodies were used: Oct-4, 1:200 (mouse monoclonal, Santa Cruz Biotechnology); mouse nestin, 1:50 (rabbit polyclonal, McKay Lab); human nestin, 1:100 (mouse monoclonal, R&D Systems); engrailed-1, 1:1000 (polyclonal, kindly provided by Dr. A. Joyner); TuJ1, 1:500 (mouse monoclonal, Covance); TH, 1:400 (rabbit polyclonal, Pelfreez); lmx1b, 1;1000 (rabbit polyclonal, kindly provided by Dr. T. Jessell); GATA4, 1:100 (rabbit polyclonal, Santa Cruz); Brachyury, 1:1000 (rabbit polyclonal, kindly provided by Dr. J. Smith); Mixl1, 1:200 (rat monoclonal); AFP, 1:500 (mouse monoclonal, Sigma); Sox1, 1: 500; cdx2, 1: 50 (mouse monoclonal, BioGenex); VASA, 1:1000 (rabbit polyclonal); SSEA1, SSEA4 and HB9 1:50 (mouse monoclonal Iowa Hybridoma Bank, MC-480, MC813-70 and 81.5C10); placental alkaline phosphatase, 1:500 (mouse monoclonal, Chemicon); OV6, 1:100 (mouse monoclonal); cytokeratin 8, 1:200 (mouse monoclonal, Sigma); cytokeratin 18, 1:200 (mouse monoclonal, DAKO); human albumin, 1:800 (rabbit polyclonal, Sigma); β-catenin, 1:1000 (mouse monoclonal Zymed); Synapsin 1, 1:500 (rabbit polyclonal, Molecular Probes). Appropriate fluorescence-tagged secondary antibodies (Jackson ImmunoResearch Laboratories or Molecular Probes) were used for visualization. GGT activity staining was performed as described earlier (Zaret et

al., *Dev. Biol.* 209, 1-10, 1999). Quantitative immunocytochemical data were expressed as means ± S.E.M.

RNA extraction and RT-PCR analysis: Total cellular RNA extraction and RT-PCR were carried out using standard protocols as described as previously (Ying et al., *Nat. Biotechnol.* 21:183-186, 2003). Forward and reverse primer sequences, annealing temperature and the number of cycles were as follows: Brachyury: 5'-taaggtggatcttcaggtagc-3' (SEQ ID NO: 1), 5'-catctcattggtgagctccct-3' (SEQ ID NO: 2) (57°C, 32 cycles); β-actin: 5'-tggcaccacaccttctacaatgagc-3' (SEQ ID NO: 3); 5'-tggcaccacaccttctacaatgagc-3' (SEQ ID NO: 4)(57°C, 32 cycles).

Flow cytometry analysis: Undifferentiated hES were harvested, separated from MEF cells as described above, and dissociated into single cell suspensions using 0.05 % Trypsin/EDTA solution. For SSEA-4 staining, unfixed cells were washed in PBS + 2 % FBS three times and stained with SSEA4 antibody (Iowa Hybridoma Bank, MC813-70, 1:50) or an IgG isotype control for 30 minutes at 4°C followed by staining with an anti mouse IgG Alexa Fluor® 488 conjungated secondary antibody (Molecular Probes). For intracellular Oct-4 staining (mouse monoclonal anti Oct-4, Santa Cruz, 1:20) cells were fixed and stained with the CytoFix/CytoPerm™ Kit (BD Bioscience) according to manufactures instructions. Flow cytometry analysis was performed on a FACSCalibur (BDIS) and Cell Quest software (BDIS).

Reverse-phase HPLC determinations of catecholamine: At day 8 of stage 5, catecholamine levels were determined in the conditioned medium (48 hours after medium change). After 15 days of differentiation, dopamine release was evaluated in modified Krebs HEPES buffer solution in the presence or absence of 56 mM KCl (evoked release, 15 minute incubation). The buffers were collected, immediately stabilized with orthophosphoric acid (7.5%) / metabisulfate (0.22 mg/ml), and stored at -80°C until analysis. Aluminum absorption, equipment, and HPLC analysis have been described previously (Ying et al., *Nat. Biotechnol.* 21:183-186, 2003). Results

were normalized against catecholamine standards at varying flow rates and sensitivities.

Electrophysiology: For electrophysiological recordings, cells were grown over confluent glial beds In N2 medium supplemented with GDNF (20 ng/ml), BDNF (20 ng/ml), NT-3 (20 ng/ml), NGF (50 ng/ml) and 10% serum. Coverslips were transferred into a chamber continuously circulated with artificial cerebral spinal fluid recording media (124 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgSO_4$, 10 mM glucose, pH 7.4, 320 mOsm) bubbled with a 95% air, 5% CO_2 gas mixture. Patch pipettes (0.75 mm internal diameter), with resistances ranging from 8 – 20 MΩ were filled with intracellular solution (120 mM potassium gluconate, 0.2 mM EGTA, 2 mM Mg-ATP, 0.3 mM GTP-Tris, 10 mM HEPES). Recordings were performed under visual guidance using an upright microscope. Whole cell voltage clamp recordings were performed at a holding potential of -70 mV and signals were amplified using an Axopatch 200B (Axon Instruments, Union City, CA) amplifier. Current clamp recordings were performed using a second amplifier, Axoclamp 2B (Axon Instruments). All data were digitized via a Digidata 1200B (Axon Instruments) and acquired and analyzed on a PC running pClamp8.1 (Axon Instruments). To identify recorded cells, cells were loaded with the retrograde fluorescent tracer biocytin-alexa488 during voltage clamp recordings, subsequently fixed and stained with anti-Alexa Fluor® 488 (rabbit polyclonal), Tuj1 (mouse monoclonal, Covance) and TH (sheep polyclonal, Pelfreez).

Transplantation: Adult female Sprague–Dawley rats lesioned with 6-OHDA were purchased (Taconic Farms). Human ES derived dopaminergic neurons were harvested as cell clusters using HBSS solution (Stage 5a, day 8). For co-transplantation experiments, confluent mouse glia cells two weeks after preparation from E16.5 mice were trypsinized and mixed with human ES cell derived dopaminergic neurons (at an ratio neurons to glia 4 to 1) and re-suspended at a density of 160,000 viable cells per µl. Three µl of the cell suspension were grafted into the lesioned striatum of hemiparkinsonian rats (0.0 mm anteroposterior, 3.0 mm

mediolateral and 5.0 mm dorsoventral of bregma and the meninges). All animals were immunosuppressed with cyclosporine A (Neoral, Novartis, 10 mg/kg per day, intraperitoneally) starting 24 hours before grafting. The number of animals studied was six animals without and 10 animals with mouse glia. At 5 to 95 days after transplantation, rats were perfused transcardially with PBS followed by 4% paraformaldehyde. Perfused brains were postfixed overnight (4°C), soaked in 20% sucrose overnight, frozen in isopentane cooled by solid CO_2 and cut on a cryostat at 35 μm. Immunostaining was carried out using standard protocols for free floating sections. The following antibodies were used: TuJ1, 1:2000 (rabbit polyclonal, Covance); TH, 1:400 (rabbit polyclonal, Pelfreez); human nuclear antigen, 1:300 (mouse monoclonal, Chemicon); human specific 70 kDa neurofilament 1: 50 (mouse monoclonal, Chemicon); NeuN 1:200 (mouse monoclonal, Chemicon) and GFAP 1:500 (rabbit polyclonal, DAKO).

Karyotypic analysis: Cells were grown on DMEM, 10% FBS, supplements. Metaphase chromosomes were prepared from human ES cells grown on foreskin feeder-layers. Cell division was blocked by using colcemid (10μg/ml, for 30 minutes, Roche Company). Cells were treated with the hypotonic solution, KCl (0.075 M) for 15 minutes. The cells were then washed three times with fixative (3:1 methanol : Acetic Acid). Slides were aged for one week. One hundred metaphase spreads were analyzed using the molecular cytogenetic technique, spectral karyotyping (SKY). The SKY chromosome painting probes were generated from flow sorted human chromosomes using DOP-PCR DNA amplification. Spectral karyotyping (SKY) hybridization methods were performed according to previously published methods (Castrillion et al., *Proc Natl Acad Sci USA* 97: 9585-9590, 2000). The indirectly labeled biotin probe sequences were visualized using Avidin Cy5 (Jackson Immuno Research Labs), and the digoxygenin-labeled probe sequences by incubation with mouse anti-digoxin antibody (Sigma-Aldrich) followed by a sheep anti-mouse-antibody conjugated to Cy5.5 (Jackson Immuno Research). Chromosomes were counterstained with DAPI and the antifade, paraphenylene-diamine (Sigma-Aldrich) was applied. Image acquisition was performed using a SD200 Spectracube (Applied Spectral Imaging, Inc.) mounted on a Leica DMIRBE

microscope using a custom designed optical filter (SKY-1, Chroma Technology). Chromosome aberration nomenclature followed the rules found in ISCN (1995, Mittleman).

FM 4-64 optical imaging: All steps were performed in modified Tyrode's solution containing (in mM): 150 NaCl, 4 (normokalemic) or 50 (hyperkalemic) KCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, and 0 or 2 $CaCl_2$, pH 7.4 (320 mOsm). Hyperkalemic solutions contained an equimolar substitution of KCl for NaCl. Synaptic terminals were loaded with 15 μM FM4-64 (Molecular Probes, Eugene, OR) in buffer containing 50 mM KCl for 1 minute followed by washing in dye-free, 0 mM $CaCl_2$ solution for 10 minutes. All staining and washing protocols were performed with 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 50 μM aminophosphonopentanoic acid (AP-5) to prevent recurrent activity. Coverslips with loaded cells were transferred into sealed imaging chamber mounted under Zeiss 510 confocal microscope. Images were taken after 15 minute starting from initial loading. The field with apparent neuronal processes was selected for imaging. Destaining of neuronal terminals with hyperkalemic buffer was achieved by peristaltic pump perfusion of solutions onto the chamber (2 ml/minute). FM4-64 was excited by HeNe laser at 543 nm and 55 serial images (3 Z-sections) were acquired every 5 seconds using LSM 510 software. Images were analyzed with MetaMorph Software (Universal Imaging, Downingtown, PA). To determine the level of photobleaching we followed fluorescence loss from areas that neither did not release dye nor were associated with neuronal processes (baseline). Using MetaMorph software average gray values were measured of fluorescent puncta that were clearly localized to neuronal processes. The (ΔF/F) for each puncta over time was measured after subtracting last image. Delta F was normalized to initial fluorescence value (ΔF/F).

Example 7

Differentiation to neurectoderm

Immunocytochemistry and FACS analysis for the expression Oct4 and

SSEA4 and SKY karyotyping established that that the starting cells (human ES cell line HSF-6) are undifferentiated and have a normal set of chromosomes (Fig. 4; NIH # UC06, Passage 38-120). Cell aggregation promotes differentiation to many somatic cell types but there is little systematic data on the cell types in these aggregates known as embryoid bodies (EBs) for mouse or human ES cells. Highly enriched preparations of functional midbrain dopaminergic neurons are essential for research on Parkinson's disease and have not been obtained from human ES cells.

A protocol has been developed for the differentiation of mouse ES cells into neural precursors and dopaminergic neurons that function *in vivo* (Kim et al., *Nature* 418: 50-56, 2002). This method relies on generating EBs containing large numbers of neural precursors and subsequent selection and expansion of midbrain precursors in the presence of morphogens that promote midbrain fates. However, the differentiation of human ES cells has not been demonstrated using this protocol.

Sox1 and nestin, genes are normally expressed throughout the developing nervous system. After the formation of human EBs, many cells express these genes suggesting efficient differentiation to neurectoderm (Fig. 4D). These Sox1 and nestin positive EBs were plated after 8 days of aggregation on adherent tissue culture dishes in ITSFn medium to promote cell attachment. Over the course of an additional 8 days, a cell population containing many nestin positive precursors migrated out of the EBs. These cells were expanded after dissociation and re-plating on poly L-ornithine coated plates in the presence of FGF-2. Terminal differentiation was induced by FGF2 withdrawal. Under these conditions 10 to 20 % of the cells adopted a neuronal morphology and expressed neuron specific tubulin Tuj1 (Fig. 4).

An important advantage of the ES cell strategy is that the neurons with different identities can be obtained by applying developmentally appropriate signals to the differentiating CNS stem cells. In the presence of FGF-4, the number of Sox1-positive cells after aggregation increased from 45.31 +/- 7.08 to 74.59 +/- 3.91% (for example, see Fig. 4). The number and size of the FGF-4 treated EBs was significantly larger than controls suggesting that FGF-4 promotes neural precursor proliferation and/or survival. When the FGF-4 treated EBs were transferred to an adherent surface, a homogeneous population of nestin positive neural precursors containing multiple rosette forming structures developed (Fig 4D). After FGF-4

treatment, the number of TuJ1 positive cells with neuronal morphology increased to 60.9 +/- 5.9% (Fig. 4A-4B). $GFAP^+$ or $O4^+$ astrocytes and oligodendrocytes were also seen. FGF-2 or FGF-8 treatment of EBs lead only to a minor increase of TuJ1 positive neurons (Fig. 4B). These results show that highly enriched populations of neural precursors can be obtained *in vitro* from human ES cells.

Example 8

Differentiation to dopamine neurons

The diffusible morphogens sonic hedgehog (Shh) from the floor plate and FGF-8 produced by the isthmic organizer contribute to the patterning of the mid- and hindbrain. Many nestin–positive cells were identified after treatment with FGF-4 at the EB and attachment steps that also expressed the transcription factor engrailed-1. Unperturbed maturation of FGF4 treated populations led to the identification of tyrosine hydroxylase (TH)-positive dopamine neurons. Because TH is an enzyme crucial for the production of the neurotransmitter dopamine in the substantia nigra midbrain neurons that are lost in Parkinson's disease, optimization of the yield of TH+ dopamine neurons was sought using Shh/FGF-8 treatment. The proportion of TH+ neurons increased from 23 +/-4% to 58 +/- 9%. The LIM homeodomain protein, Lmx1b, is necessary for the induction of the homeodomain gene Ptx3 in midbrain dopamine neurons and is also necessary for the survival of these cells in the mouse (Johe et al., *Genes Dev.* 10:3129-3140, 1996). Following treatment with Shh/FGF8, over 90% of the TH^+ neurons also expressed Lmx1b.

To further confirm that appropriate signals regulate the identity of neurons generated from ES cells, human ES cells were treated with retinoic acid (RA). RA treatment of differentiating human ES cells induced HB9+/Sox1 co-expressing precursors, whereas after FGF4 treatment in the absence of RA, no HB9 positive precursor cells were observed. After terminal differentiation, TuJ1 positive neurons co-expressing HB9 were abundant. None of the neurons after RA treatment expressed TH. These responses to SHH, FGF8 and RA suggest that appropriate physiological signals regulate the positional identity of precursors and neurons generated from human ES cells.

Neuronal function requires membrane recycling and vesicle fusion. The styryl dye FM4-64, labels synaptic vesicles in neurons an activity-dependent manner and provides an assay to image vesicle recycling over many cells at the level of individual synaptic terminals (Vicario-Abejon et al., *Eur J Neurosci.* 12, 677-88, 2000). Antibodies against proteins found in synaptic vesicles identify punctate structures in the ES-derived neurons suggesting the formation of synaptic sites (Fig. 5). To depolarize a population of neurons and stimulate calcium influx, a buffer containing 50 mM KCl was applied, eliciting release of dye with accelerated kinetics from a portion (~25%) of FM4-64 positive puncta (Fig. 5). This result suggests that the human ES cell-derived neurons are capable of Ca^{++}-dependent synaptic vesicle release.

Histochemical data demonstrated that midbrain-type dopamine neurons were differentiated from human ES cells. Reverse phase HPLC was used to measure the concentrations of L-DOPA, DA and the DA metabolite, DOPAC (Fig. 5A). In the non-treated, control cultures, the concentrations of these compounds were 274.9 ± 22.8 pmol/ml, 4.98 ± 0.5 pmol/ml and 4.13 ± 0.67 pmol/ml (mean ± S.E.M.), respectively. When the cultures were treated with Shh and FGF-8 in stage 4 the values increased significantly ($p<0.01$) up to 481.2 ± 22.8 pmol/ml, 17.74 ± 1.2 pmol/ml and 27.14 ± 2.62 pmol/ml. Noradrenaline and adrenaline were not observed in these cultures using an assay with a detection limit of 320 fmol/ml and 360 fmol/ml, respectively. In response to a depolarizing stimulus (50 mM K^+), ES cell–derived neurons released dopamine into the culture medium (Fig. 5B). These data suggested that the neurons are indeed capable of synthesizing and releasing the neurotransmitter dopamine.

The ability to fire action potentials, especially repeated trains of action potentials, is essential for activity-evoked release of transmitter from neurons. Patch-clamp recording methods were used to monitor the electro-physiological maturation of human ES cell-derived neurons as they differentiated. Neuronal differentiation can be synchronously triggered by withdrawing mitogens supporting the proliferation of CNS stem cells. Neurons differentiated from human ES cells by mitogen withdrawal without further manipulation were electrically inert and non-excitable. Neurons derived human ES cells 5-9 days after mitogen withdrawal were

re-plated onto astrocytes derived from the midbrains of E16.5 mice in media supplemented with GDNF (20 ng/ml), BDNF (20 ng/ml), NT-3 (20 ng/ml), NGF (50 ng/ml) and 10 % fetal calf serum. Voltage- and current clamp-recordings revealed the gradual development of voltage-dependent inward sodium currents and distinct outward, delayed rectifier potassium currents. The voltage-clamp recordings indicate that the neurons initially expressed only non-rectifying potassium currents until the point when the cells became electrically excitable and capable of action potential propagation (Fig. 7). The onset of evoked, repetitive action potentials corresponded with the expression of distinct outward currents (Fig. 7C at 22-24 days), specifically delayed rectified potassium currents (Fig. 7F). Pharmacological perturbation reveals that the inward currents are TTX-sensitive sodium currents and sequential application of 4-aminopyridine, a selective blocker of A-type potassium channels, inhibited the rapidly inactivating, A-type potassium currents (Fig. 7F).

Delayed rectifier potassium channels, including members of the Kv1, Kv2, Kv3 and Kv4 subfamilies, modulate action potential duration, repetition and associated calcium influx. Slowly activating I_{Kv} currents are characteristic of immature neurons from many species (Vicario-Abejon et al., *Eur J Neurosci.* 12:677-88, 2000). Acceleration of I_{Kv} kinetics corresponds with reduction of calcium dependence and maturation to a rapid, sodium-dependent action potential that can be repetitively generated. Consistent with this developmental program, acceleration of I_{Kv} kinetics was observed throughout the course of human ES cell-derived neuronal differentiation. The acceleration of delayed rectifier potassium currents is gradually masked by the expression of rapidly inactivating 4-AP A-type potassium currents (Fig. 7C, arrow; Fig. 7F). At this stage, neurons are capable of firing repeated, but decrementing action potentials. The ability to fire tonically may be hindered by the continued expression of slowly activating delayed rectifier potassium currents at this stage (Fig. 7F). In the next step (28-30 days), the kinetics of potassium currents accelerated to a mature phenotype and depolarizing current pulses evoked repetitive firing of action potentials (Fig. 7D). Importantly, at this stage current clamp recordings first revealed spontaneous synaptic activity in neurons maintained at a resting potential of -30 mV (Fig. 7B, 7C).

To specifically assess the physiological properties of human ES cell-derived dopamine neurons, cells were loaded with the retrograde fluorescent tracer biocytin-alexa488 during voltage clamp recordings and subsequently fixed and the expression of TH, Tuj1 and biocytin were analyzed. TH+ and TH- neurons displayed similar ionic properties (Fig. 7G), with voltage-dependent ionic currents and action potential generation. At longer periods of incubation (23+ days), 85% of all the TH+ and 80% of the TH- neurons recorded (n=12 TH+/Tuj+; N=6 TH-/Tuj1+) were synaptically active (Fig. 7G). Dopamine neurons of the substantia nigra have properties that are unique compared to other neuron types in the substantia nigra, including a hyperpolarization-induced (I_H) current, which facilitates rhythmic firing and pacemaker activity that are both used to identify the cells in slices of adult brain. None of the TH+ or TH- neurons displayed an active I_H current (Fig. 7G). These results suggest that although dopamine neurons can be efficiently generated, the acquisition of this property does not occur in the present culture conditions. These data clearly establish that physiologically active but immature TH+ neurons are efficiently generated from human ES cells. Lack of appropriate cells is one of the likely causes for the poor results in two recent clinical trials where fetal midbrain tissue was transplanted to Parkinson's patients (Lindvall, *Pharmacol. Res.* 47:279-287, 2003).

The expression of region specific genes and neuronal functions suggests that the appropriate type of neuron for cell replacement therapy in Parkinson's disease can be generated from human ES cells. In rodents, administration of 6-hydroxy dopamine (6-OHDA) in the striatum specifically kills dopamine neurons, providing a useful model of Parkinson's disease. After transplantation of 5 X 10^5 human ES cell derived dopaminergic neurons (day 8 after differentiation) into the dorsal striatum of 6-OHDA lesioned animals, no surviving TH positive neurons were found 3 days to 2 weeks after transplantation. To facilitate local environmental support for grafted human ES cell derived dopamine neurons, ventral midbrain astrocytes derived from E16.5 mice were co-transplanted. The co-transplant of midbrain glia with human ES cell derived dopamine neurons promoted the survival of TH+ dopamine neurons. Under these conditions, multiple clusters of TH expressing cells with the morphology of immature neurons could be found 5 days after

transplantation. At later timepoints (up to 95 days after transplantation), TH+ neurons were not identifiable. However, large human grafts were found in the striatum for up to 95 days after transplantation. Many human neurons expressing Tuj1, NeuN and human specific 70kDa neurofilament (Figs. 4 I, 4L, 4M, 4N, 4O) were found in these grafts. These results suggest that transplanted human ES cell-derived dopamine neurons are present initially in the presence of supporting cues from co-grafted midbrain glia but do not survive.

Example 9

Differentiation of endoderm and hepatocytes

Because of their putative pluripotency, human ES cells promise access to many cell types but there are few examples where cells have been isolated in large numbers with high purity. As disclosed herein, patterns of gene expression characteristic were used to explore the potential of human ES cells in order to generate other cell types of the inner cell mass, the structure that gives rise to the embryo itself that at early stage is known as the epiblast. The early embryo contains embryonic cells and the extra-embryonic cells of the primitive endoderm. In the mouse, these extra-embryonic cells are comprised of parietal and visceral endoderm. They can be easily recognized in the embryo and in EBs because they envelop the epiblast and express both a-fetoprotein (AFP) and the transcription factor, GATA4. In the human embryo, the primitive endoderm is called the hypoblast and the distinction into parietal and visceral types is not so well defined. In contrast to the mouse, there are few AFP+GATA4+ presumptive hypoblast cells at the surface of untreated human EB's (Fig 6A). Lithium activates the wnt signaling pathway (Klein & Melton, *Proc Natl Acad Sci USA* 93:8455-8459, 1996). When EBs were treated with lithium (10mM LiCl) from day 2-4 and analyzed at day 8 of cell aggregation, there was a greater than 10-fold increase in the number of AFP+GATA4+ cells forming a surface layer on almost all human EBs (Figs. 6B, 6D). Nuclear beta-catenin identifies cells engaged in wnt signaling and was found in EBs containing AFP+GATA4+ cells at the surface (Fig 6B - inset). Membrane bound beta-catenin was found in all differentiated human EBs. These results suggest that Li^{++} treatment induces sustained wnt signaling in the differentiation of hES cells towards the

hypoblast fate. Analysis of mice nullizygous for wnt-pathway genes suggests that wnt signaling is necessary for the signals from the visceral endoderm that specify the node, a key organizing center for gastrulation (Ishikawa et al., *Dev. Biol.* 253:230-246, 2003). The results presented herein suggest wnt signaling can promote the formation of extra-embryonic endoderm itself in human embryonic development.

GATA4+ and AFP- cells were present internally in untreated EB's and the number of these cells was also significantly increased by treatment with lithium (Fig. 6D). Treatment with fetal bovine serum also increases the number of GATA4+ cells within EBs (Figs. 8C, 8D). In serum-treated EBs, few AFP+, GATA4+ cells were seen suggesting that extra-embryonic fates were not efficiently generated. In the mouse embryo, GATA4+, AFP- cells are progenitors of the cardiac and definitive endoderm lineages (Soudais et al., *Development* 121: 3877-3888, 1995). EBs generated in the presence of fetal calf serum contained organized structures composed of epithelia that express markers found in the primitive gut when the anlage of endodermal organs first forms. The transcription factors Hnf3β (also known as Foxa2) and Cdx2 are co-expressed in the caudal endoderm that gives rise to the intestinal system. Many HNF3β,Cdx2 double positive epithelial structures were found in the EBs (Fig. 6E-H). HNF3β+, Cdx2- epithelia were also found (Fig. 6I). Epithelia of this type would be consistent with differentiation to rostral endodermal fates including liver, pancreas and lung. HNF3β+Pax6+ and HNF3β+Isl1+ epithelial structures were also seen within the cystic EBs (Fig. 6J and 6K). These patterns of expression are found in developing pancreatic endoderm; these data suggest that the GATA4+, AFP- cells contain early precursors of different endodermal organs.

In order to determine that these precursor cell types in the EBs are capable of generating endodermal fates, human EBs treated with lithium or serum were re-plated into dishes that support cell attachment. Over the ensuing 8 days, large areas of the surface were covered by a homogeneous population of polygonal cells that migrated away from the EBs. After 4 days, many of these polygonal cells expressed markers of fetal hepatoblasts (GATA4, HNF3β, OV6, CK8. CK18 and albumin).

Differential expression of keratins distinguishes the earliest hepatic precursors from more differentiated cells. Primitive hepatocytes and adult oval cells are identified by the OV6 antibody (Ruttenber et al., *J. Histochem. Cytochem.* 17:517-526, 1969), which recognizes cytokeratins 14 and 19, and by cytokeratin 8 expression (Van Eyken et al., *Lab Invest* 59:52-59, 1988). More differentiated hepatocytes express cytokeratin 18, Hnf3b and albumin. Cells expressing these markers of a more advanced stage of hepatocyte differentiation were seen after an additional 4 days of culture. At this time, almost all of the cells express HNF3β (hepatocyte nuclear factor-3beta), cytokeratin18 and many multinucleate cells were present (Fig. 6L). In addition, a colorimetric, enzymatic assay showed that many cells were positive (~30%) for gamma-glutamyl-transpeptidase (GGT), an enzyme expressed in fetal hepatocytes and bile duct cells but not adult hepatocytes (Fig. 6M). These results suggest that the endodermal precursors in the EBs can generate immature hepatocytes in large numbers.

Example 10

Differentiation of endo-mesoderm and primitive germ cells

The T-box transcription factor brachyury, first identified in the mouse, is thought to play a critical role in the differentiation of the mesoderm during gastrulation in all species that have a trilaminar organization (Wilkinson et al, *Nature* 343:657-659, 1990). An antibody raised against Xenopus brachyury was shown to react with axial mesodermal structures in the mid-gestation mouse embryo and then applied to the human EBs (Fig. 8A). Brachyury expression was detected in few cells in EBs at 8 days under control conditions (Fig. 8C). Factors in serum are known to induce the brachyury. Mesoderm, as measured by the number of brachyury-positive cells, was induced in human EB's by serum (Figs. 8B, 8C). RT-PCR analysis shows transient induction of brachyury under control conditions and a sustained expression in serum treated EBs (Fig. 8D). Mxl is expressed during the morphogenesis of mesodermal structures, especially the node, notochord, axial mesoderm and the gut, and in the differentiation of the definitive endoderm (Mohn et al., *Dev Dyn.* 226:446-59, 2003). Nearly all Brachyury positive cells in serum treated human EBs coexpress Mixl1. In contrast, many Mixl1 positive cells do not

express Brachyury (Figs. 8E and 8F). Interestingly co-staining of serum treated EBs with Oct4 revealed that nearly all Brachyury and/or Mixl1 positive cells co-expressed Oct4 (Figs. 8E, 8G, 8H). Studies in zebrafish of pou5f1/pou2, the homolog of mammalian Oct4 suggest a role beside its function in pluripotent stem cells in the formation of endoderm by maintaining sox32/cas expression and initiation of sox17 expression (Toyooka et al., *Proc Natl Acad Sci USA* 100:11457-11462, 2003). These data provide additional evidence for the induction of the first steps in the formation of definitive endoderm and mesoderm from human ES cells.

Undifferentiated ES cells can express genes associated with the germ line and under some conditions differentiate to form cells that express many features of gametes (Geijsen et al., *Nature* 427:148-54, 2004; Hubner et al., *Science* 300:1251-6, 2003). To determine if human PGCs were generated in EBs, markers of early (AlkPhos, SSEA-) and later (VASA) embryonic germ cell development were analyzed. Alkaline phosphatase is widely used as a marker for the cells of the germ line. In the mouse embryo, AlkPhos+ and SSEA-1 are markers for newly induced PGCs while the VASA gene is expressed when migrating PGCs arrive at the genital ridge. Clusters of AlkPhos+ and SSEA1+ cells were found in restricted locations in human EBs (Fig. 9G, 9H). SSEA1+ cells were closely associated with AlkPhos+ and VASA+ cells (Figs. 8I, 8J). Consistent with previous work on mouse ES and EG cells, VASA+ cells were rarely seen in undifferentiated human ES cells but were found in many EBs treated with BMPs (Figs. 8E, 8F, 8K). BMPs induce the development of germ cells in the mouse epiblast and mouse ES cells (Ying et al., *Proc Natl Acad Sci USA* 98:7858-7862, 2001). The VASA+ cells did not express high levels of Oct4. These data suggest that the early steps in human germ cell differentiation can be studied in the EB model.

Many applications of human ES cells in medicine require the *in vitro* generation of large numbers of specific terminally differentiated cell types. It is demonstrated herein that human ES cells differentiate to distinct early cell fates, including neurectoderm, mesoderm, endoderm and primitive germ cells. These data suggest that gastrulation mechanisms occur in the absence of the precise order of the embryo. The derivation of somatic cells from these precursors shows that these precursors have the potential to generate mature cells and also that this can be a

simply achieved in a few steps. Specifically, the distribution of cell types in the EB can be controlled by providing different soluble factors. For example, exposing the EBs to serum promoted the differentiation of endoderm and mesoderm. FGF-4 and RA enhanced the production of different types of neurons. The production of dopaminergic neurons in response to FGF4 and not FGF2 differs from the previous work with mouse ES cells. In addition, there is further enrichment when the EBs are placed in conditions that support cell attachment. As demonstrated here by electrophysiological analysis of single neurons, the differentiation of the somatic cells can also occur in a highly co-operative manner.

It will be apparent that the precise details of the methods or compositions described may be varied or modified without departing from the spirit of the described invention. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

WE CLAIM:

1. A method of culturing human embryonic stem cells to produce a population of cells comprising human neuronal cells, wherein the method comprises:

expanding undifferentiated embryonic stem cells in the presence of fibroblast growth factor (FGF)-2 and dissociating the undifferentiated embryonic stem cells to form a population comprising a majority of single cells;

generating embryoid bodies from the population comprising the majority of single cells in the presence of FGF-4 and in the absence of feeder cells;

culturing the embryoid bodies in the presence of FGF-4 on an extracellular matrix to select for central nervous system precursor cells;

expanding the central nervous system precursor cells by culturing the central nervous system precursor cells in an expansion medium that comprises at least one of FGF-2, sonic hedgehog factor (Shh) and FGF-8, wherein the central nervous system precursor cells are not cultured on the extra-cellular matrix; and

differentiating the expanded central nervous system precursor cells to form a culture of differentiated neuronal cells by culturing the expanded central nervous system precursors in a culture medium that lacks FGF-4, FGF-2, Shh, and FGF-8;

thereby producing the population of cells comprising at least 30% neuronal cells.

2. The method of claim 1, wherein the extracellular matrix is fibronectin.

3. The method of claim 2, wherein the expanding the central nervous system precursor cells in an expansion medium comprises culturing the cells on a solid surface coated with poly-ornithine.

4. The method of claim 1, wherein generating embryoid bodies comprises culturing the cells for about eight days.

5. The method of claim 1, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells for about eight days.

6. The method of claim 1, wherein differentiating the expanded neuronal precursor cells comprises culturing the cells for about eight to about thirty days.

7. The method of claim 1, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells in the presence of FGF-2.

8. The method of claim 1, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells in the presence of FGF-8.

9. The method of claim 1, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells in the presence of Shh.

10. The method of claim 1, wherein the differentiated neuronal cells are dopaminergic cells.

11. The method of claim 1, wherein the differentiated neuronal cells electrically mature and exhibit synaptic vesicle release.

12. The method of claim 1, wherein expanding undifferentiated embryonic stem cells in the presence of fibroblast growth factor (FGF)-2 comprises culturing the embryonic stem cells on a feeder layer.

13. The method of claim 1, wherein expanding undifferentiated embryonic stem cells in the presence of fibroblast growth factor (FGF)-2 and generating embryoid bodies comprises culturing the embryonic stem cells and the embryoid bodies in hES medium.

14. The method of claim 1, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells in a medium comprising one or more of insulin, transferrin, and selenium.

15. The method of claim 14, wherein the medium comprises ITS medium.

16. The method of claim 1, wherein expanding the central nervous system precursor cells and differentiating the expanded central nervous system precursor cells comprises culturing in a medium comprising one or more of insulin, transferrin, putrescine, selenite, and progesterone.

17. The method of claim 16, wherein the medium comprises N2 medium.

18. The method of claim 1, wherein generating embryoid bodies and culturing the embryoid bodies to select for central nervous system precursor cells comprises culturing in the presence of about 20 ng/ml FGF-4.

19. The method of claim 1, wherein generating embryoid bodies and culturing the embryoid bodies to select for central nervous system precursor cells comprises culturing in the presence of about 20 ng/ml FGF-2.

20. The method of claim 1, wherein generating embryoid bodies and culturing the embryoid bodies to select for central nervous system precursor cells comprises culturing in the presence of about 20 ng/ml FGF-8.

21. A method of culturing human embryonic stem cells to produce a population of cells comprising human neuronal cells, wherein the method comprises:

expanding undifferentiated embryonic stem cells in the presence of fibroblast growth factor (FGF)-2 and dissociating the undifferentiated embryonic stem cells to form a population comprising a majority of single cells;

generating embryoid bodies from the population comprising the majority of single cells in the presence of retinoic acid and in the absence of feeder cells;

culturing the embryoid bodies in the presence of retinoic acid on an extra-cellular matrix to select for central nervous system precursor cells;

expanding the central nervous system precursor cells by culturing the central nervous system precursor cells in an expansion medium that comprises at least one of FGF-2, sonic hedgehog factor (Shh) and FGF-8, wherein the central nervous system precursor cells are not cultured on the extra-cellular matrix; and

differentiating the expanded central nervous system precursor cells to form a culture of differentiated neuronal cells by culturing the expanded central nervous system precursor cells in a culture medium that lacks FGF-4, FGF-2, Shh, and FGF-8;

thereby producing the population of cells comprising at least 30% differentiated neuronal cells.

22. The method of claim 21, wherein the extra-cellular matrix is fibronectin.

23. The method of claim 21, wherein generating embryoid bodies and culturing the embryoid bodies to select for central nervous system precursor cells comprises culturing in the presence of about 5 μM retinoic acid.

24. The method of claim 21, wherein expanding the central nervous system precursor cells in an expansion medium comprises culturing the cells on a solid surface coated with poly-ornithine.

25. The method of claim 21, wherein generating embryoid bodies comprises culturing the central nervous system precursor cells for about eight days.

26. The method of claim 21, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells for about eight days.

27. The method of claim 21, wherein differentiating the expanding neuronal precursor cells comprises culturing the central nervous system precursor cells for about eight to about thirty days.

28. The method of claim 21, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells in the presence of FGF-2.

29. The method of claim 21, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells in the presence of FGF-8.

30. The method of claim 21, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells in the presence of Shh.

31. The method of claim 21, wherein the differentiated neuronal cells are dopaminergic cells.

32. The method of claim 21, wherein the differentiated neuronal cells are synaptically active.

33. The method of claim 21, wherein expanding undifferentiated embryonic stem in the presence of fibroblast growth factor (FGF)-2 comprises culturing the embryonic stem cells on a feeder layer.

34. The method of claim 21, wherein expanding undifferentiated embryonic stem cells in the presence of fibroblast growth factor (FGF)-2 and generating embryoid bodies comprises culturing the embryonic stem cells and the embryoid bodies in hES medium.

35. The method of claim 21, wherein expanding the central nervous system precursor cells comprises culturing the central nervous system precursor cells in a medium comprising one or more of insulin, transferrin and selenium.

36. The method of claim 21, wherein expanding the central nervous system precursor cells and differentiating the expanded central nervous system precursor cells comprises culture in a medium comprising one or more of insulin, transferrin, putrescine, selenite or progesterone.

37. A method of culturing human embryonic stem cells to produce a population of cells comprising human neuronal cells, wherein the method comprises

expanding human embryonic stem cells on a substrate coated with an extracellular matrix in fibroblast-conditioned medium in the absence of LIF, serum, or a serum replacement;

culturing the expanded embryonic stem cells in a medium comprising one or more of insulin, transferrin and selenium in the absence of serum for at least 3 days; and

culturing the cells in a medium comprising one or more of insulin, transferrin, putrescine, selenite or progesterone in the absence of serum for at least 3 days;

thereby producing a population of cells comprising at least 30% human neuronal cells.

38. The method of claim 37, wherein the extra-cellular matrix comprises laminin.

39. The method of claim 37, wherein the medium comprising one or more of insulin, transferrin and selenium is ITS medium.

40. The method of claim 37, wherein the medium comprising one or more of insulin, transferrin, putrescine, selenite or progesterone is N2 medium.

41. The method of claim 39, wherein the expanded embryonic stem cells are culture in ITS medium for about 4 to about 6 days.

42. The method of claim 39, wherein the expanded cells are cultured in ITS medium for about 4 days, and wherein the ITS medium is replaced daily.

43. The method of claim 40, wherein the cells are culture in N2 medium for about 4 to about 6 days.

44. The method of claim 43, wherein the cells are cultured in N2 medium for about 4 days, and wherein the N2 medium is replaced daily.

45. A method of treating a neurodegenerative disorder, comprising administering to a subject a therapeutically effective amount of neuronal cells produced by the method of any one of claims 1.

46. A method of treating a neurodegenerative disorder, comprising administering to a subject a therapeutically effective amount of neuronal cells produced by the method of any one of claims 1, 21 or 37.

47. A cell produced by the method of any one of claims 1, 21, or 37.

48. A method of culturing primate embryonic stem cells to produce a population of cells comprising primate hepatocytes, wherein the method comprises:

generating embryoid bodies from a population of undifferentiated primate embryonic stem cells comprising a majority of single cells in a medium comprising one or more of fibroblast growth factor (FGF)-1, FGF-4, FGF-8, BDNF, retinouic acid;

culturing the embryoid bodies in the presence of an effective amount of a factor that activates the wnt signaling pathway;

culturing the cells in a medium comprising one or more of insulin, transferrin, or selenium and further comprising an effective amount of fibronectin or serum; thereby producing the population of cells comprising primate hepatocytes.

49. The method of claim 48, wherein the factor that activates the wnt signaling pathway comprises about 1 to about 500 mM LiCl.

50. The method of claim 48, wherein the factor that activates the wnt signaling pathway comprises about 5 to about 100 mM LiCl.

51. The method of claim 48, wherein the factor that activates the wnt signaling pathway comprises about 10 to about 50 mM LiCl.

52. A method of culturing primate embryonic stem cells to produce a population of cells comprising primate mesodermal cells, wherein the method comprises:

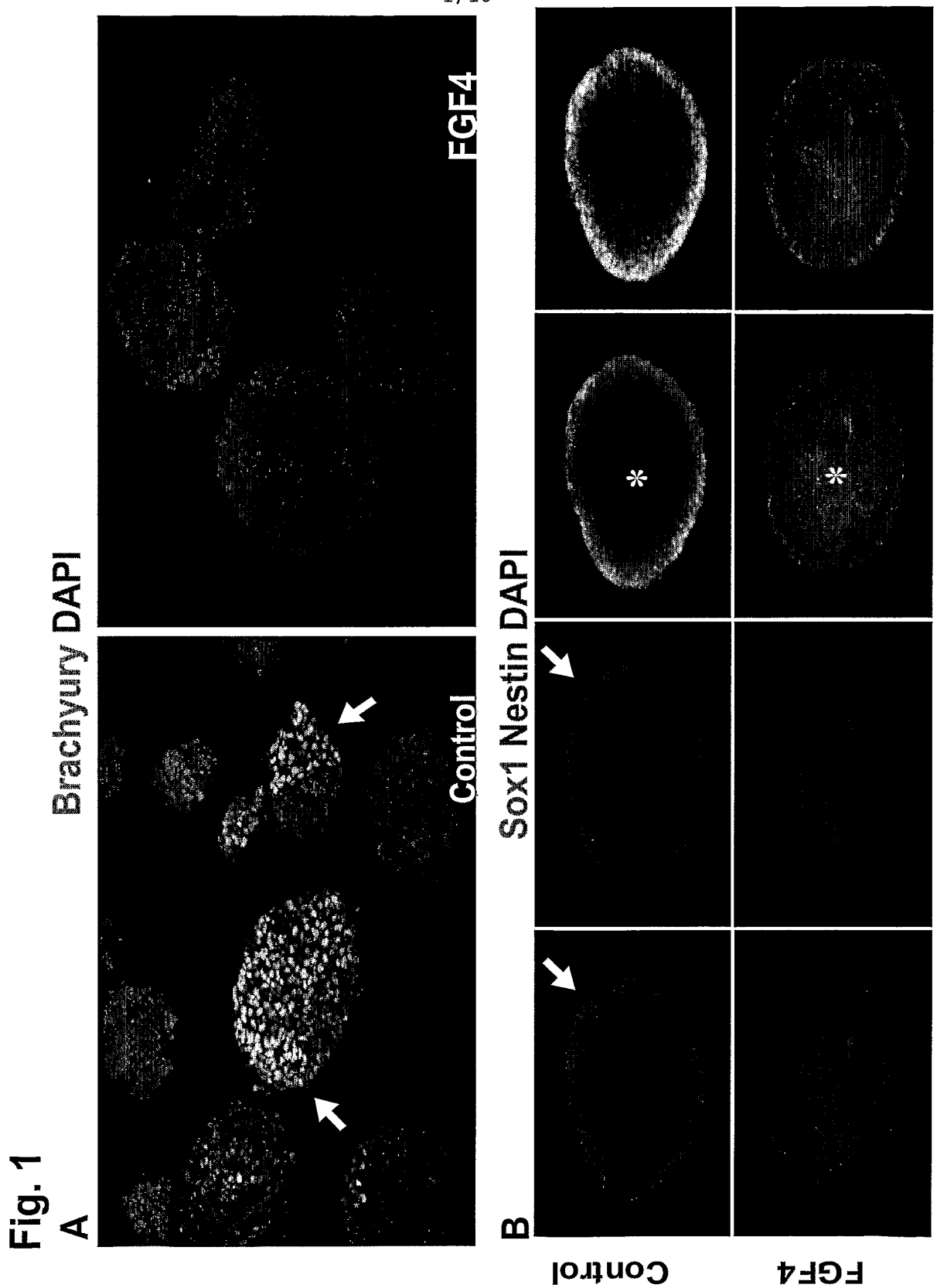
expanding undifferentiated primate embryonic stem cells in a medium comprising an effective amount of fibroblast growth factor (FGF)-2;

generating embryoid bodies from a population of undifferentiated primate embryonic stem cells comprising a majority of single cells in a medium comprising an effective amount of FGF-4 and an effective amount of a factor that activates the wnt signaling pathway;

culturing the embryoid bodies on an extracellular matrix, thereby producing mesodermal cells.

53. The method of claim 52, wherein the wnt activating factor is lithium or serum.

Fig. 1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Brachyury DAPI
Control
FGF4
B
Sox1 Nestin DAPI
Control
FGF4

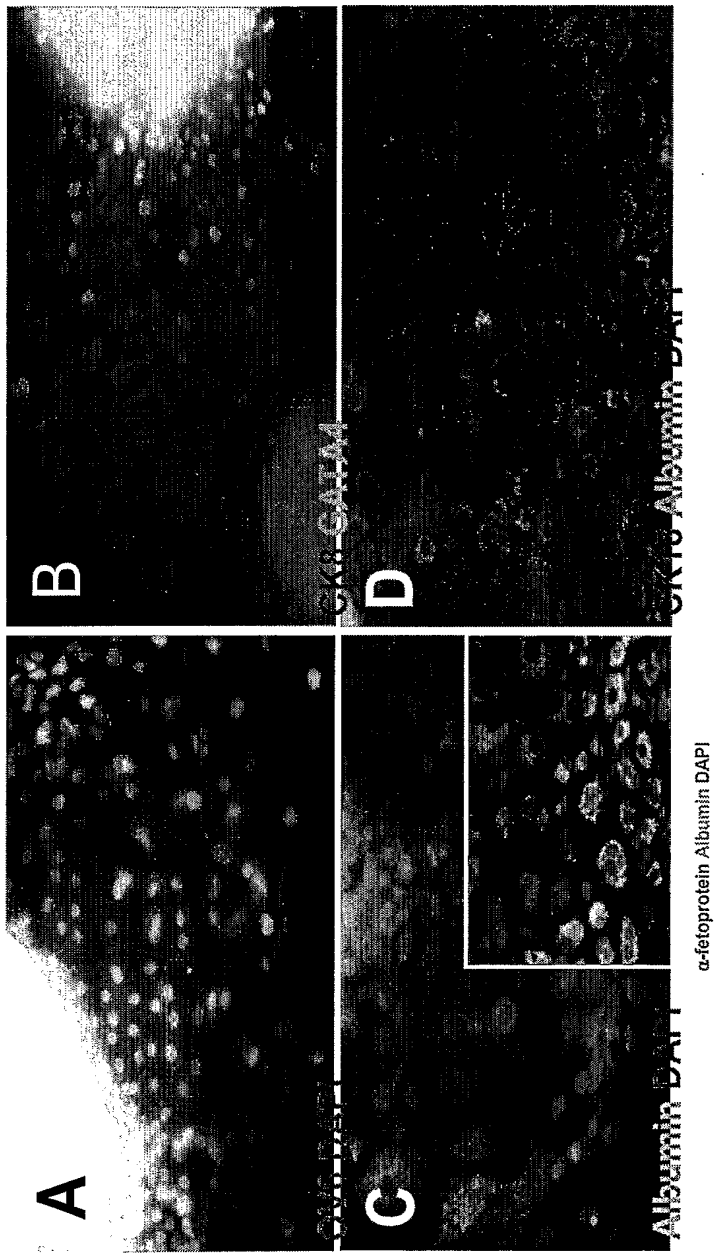

A
B
CK8 SATB1
C
Albumin DAPI
D
CK18 Albumin DAPI
α-fetoprotein Albumin DAPI
Stage 3 day 6 Stage 3 day 2

Fig. 2

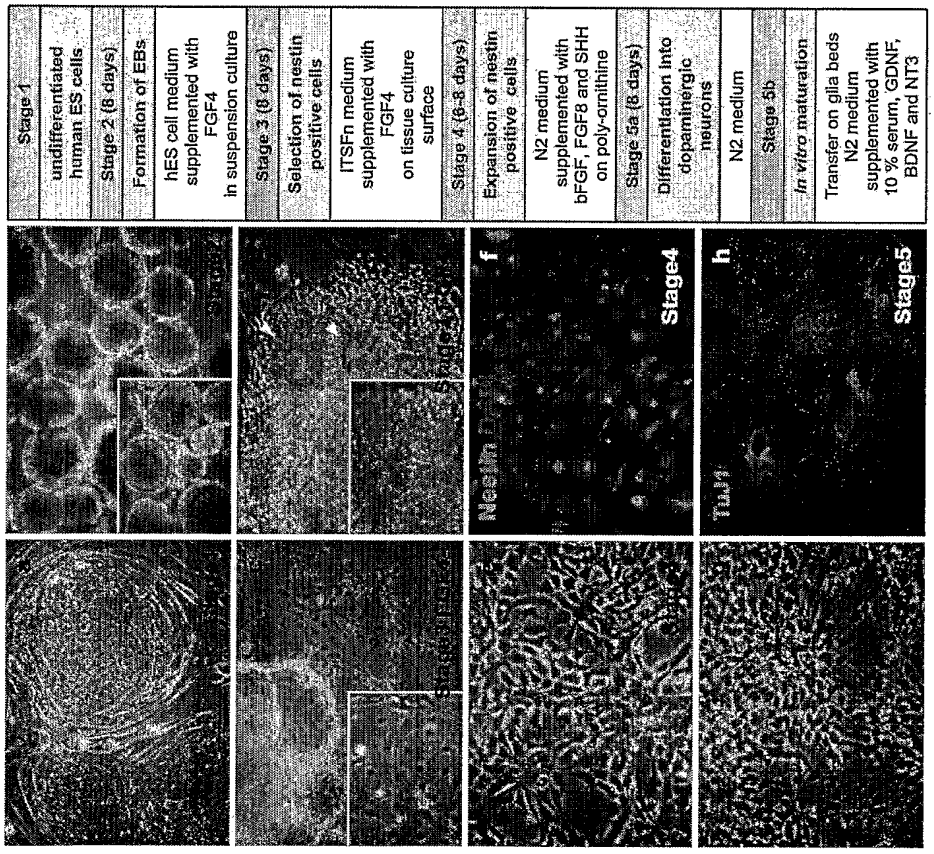

Stage 1
undifferentiated human ES cells
Stage 2 (8 days)
Formation of EBs
hES cell medium supplemented with FGF4 in suspension culture
Stage 3 (8 days)
Selection of nestin positive cells
ITSFn medium supplemented with FGF4 on tissue culture surface
Stage 4 (6-8 days)
Expansion of nestin positive cells
N2 medium supplemented with bFGF, FGF8 and SHH on poly-ornithine
Stage 5a (8 days)
Differentiation into dopaminergic neurons
N2 medium
Stage 5b
In vitro maturation
Transfer on glia beds N2 medium supplemented with 10 % serum, GDNF, BDNF and NT3
Stage1
Stage3
Nestin
f
Stage4
TuJ1
h
Stage5

Fig. 3

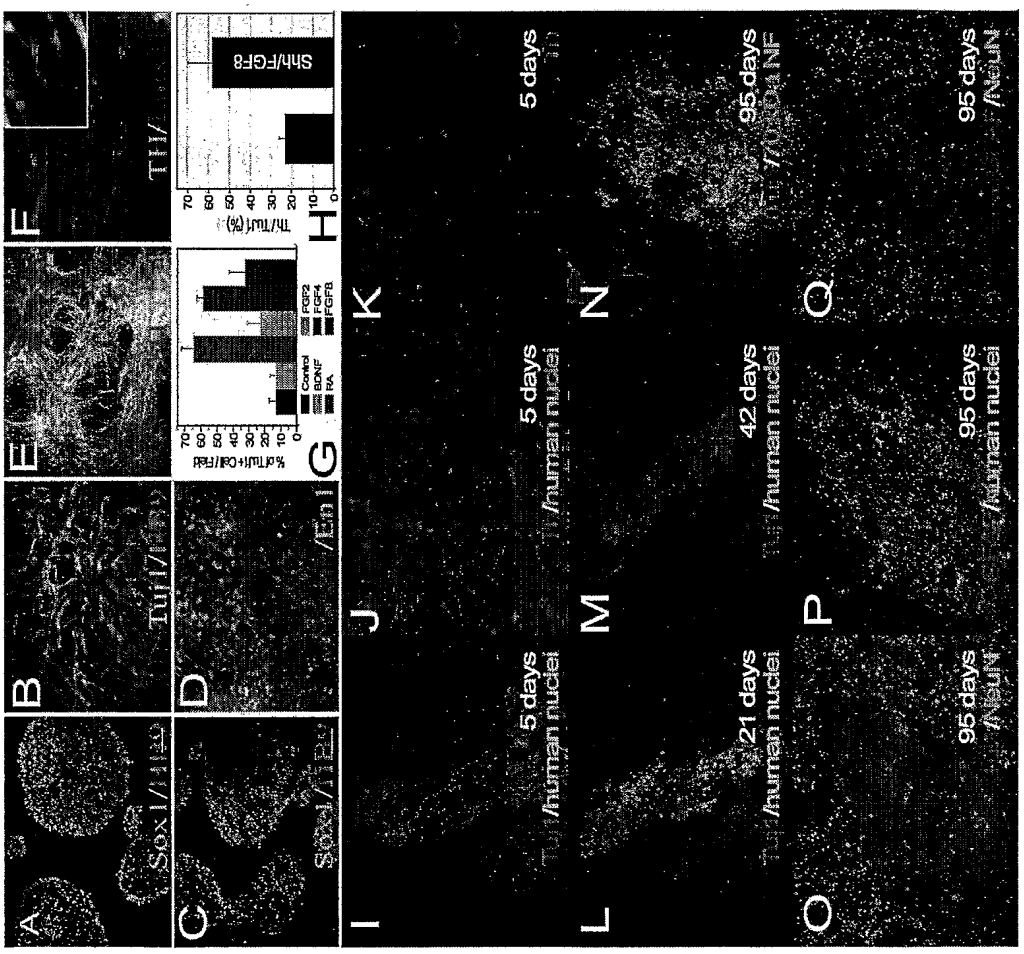
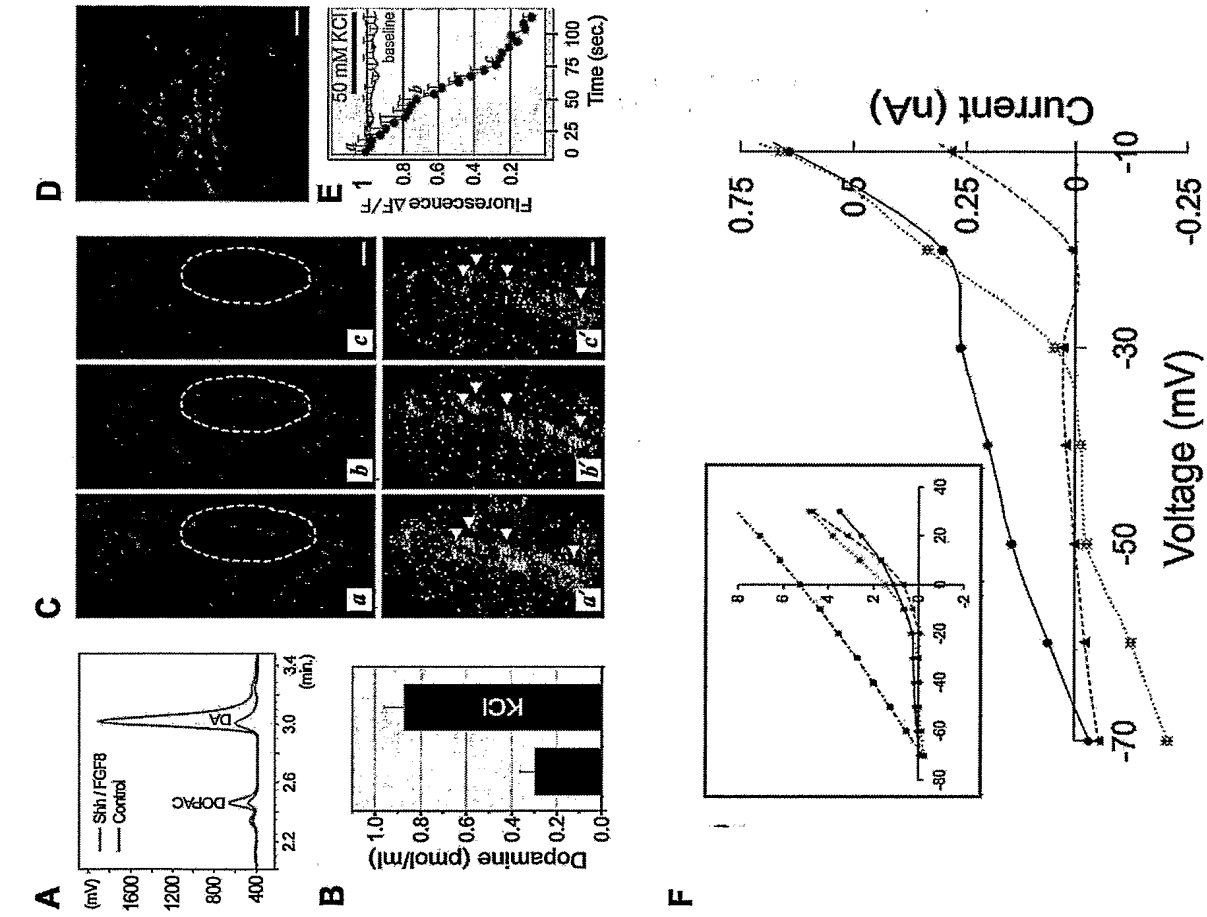

A
Sox1/Tuj1
B
Tuj1/Hoechst
C
Sox1/En1
D
/En1
E
F
TH/
G
% of Tuj1+ Cell/Field
Control
BDNF
RA
FGF2
FGF4
FGF8
H
Th/Tuj1 (%)
Shh/FGF8
I
J
K
5 days
/human nuclei
L
M
N
5 days
/human nuclei
5 days
O
P
Q
21 days
/human nuclei
42 days
/human nuclei
95 days
/NeuN
95 days
/human nuclei
95 days
/NeuN

Fig. 4


A
B
C
D
E
F
Shh / FGFB
Control
DOPAC
DA
(mV)
1600
1200
800
400
2.2
2.6
3.0
3.4
(min.)
KCl
Dopamine (pmol/ml)
1.0
0.8
0.6
0.4
0.2
0.0
50 mM KCl
baseline
Fluorescence ΔF/F
Time (sec.)
0 25 50 75 100
Current (nA)
Voltage (mV)
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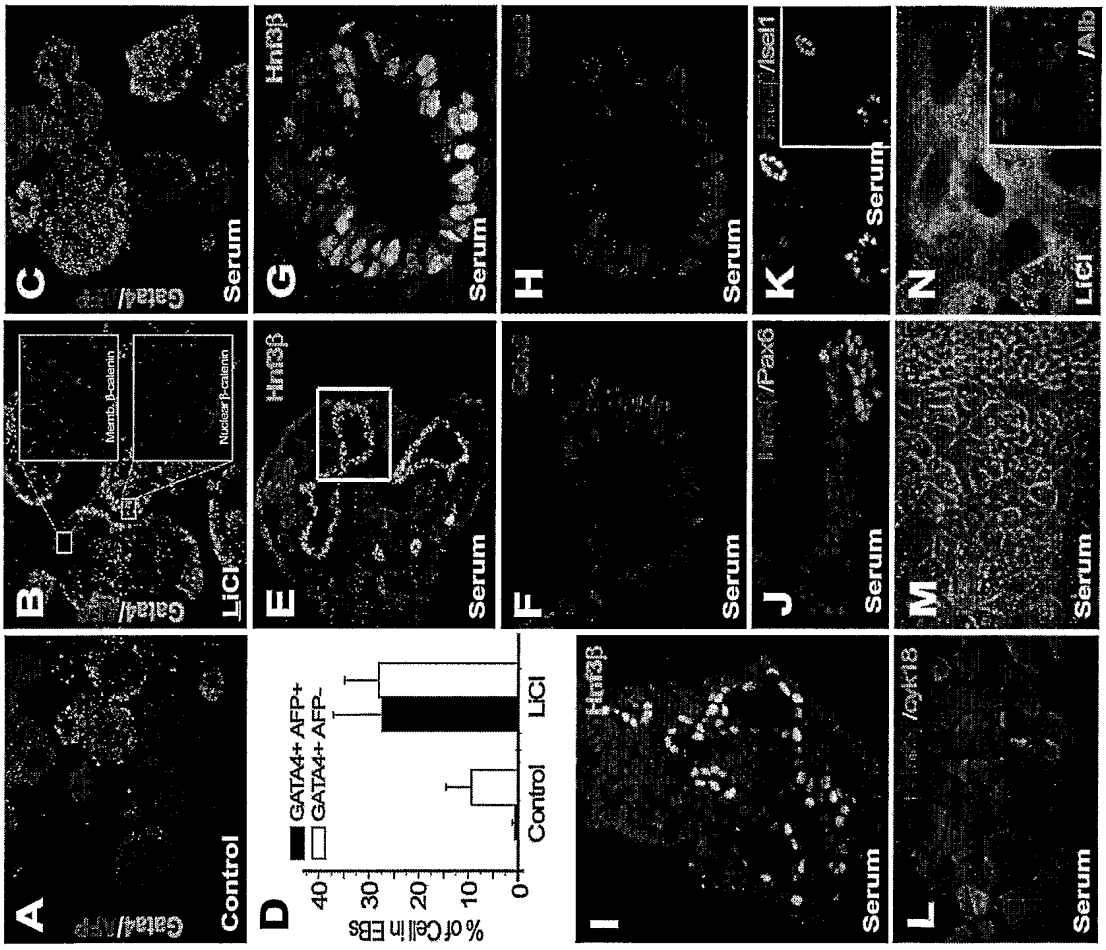
0.25
0
-10
-30
-50
-70
-0.25

Fig. 5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Gata4/AFP
Control
B
Gata4/
LiCl
Membr. β-catenin
Nuclear β-catenin
C
Gata4/
Serum
D
GATA4+ AFP+
GATA4+ AFP-
% of Cell in EBs
40
30
20
10
0
Control
LiCl
E
Hnf3β
Serum
F
Serum
G
Hnf3β
Serum
H
Serum
I
Hnf3β
Serum
J
/Pax6
Serum
K
/Isl1
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L
/cytk18
Serum
M
Serum
N
/Alb
LiCl

Fig. 6

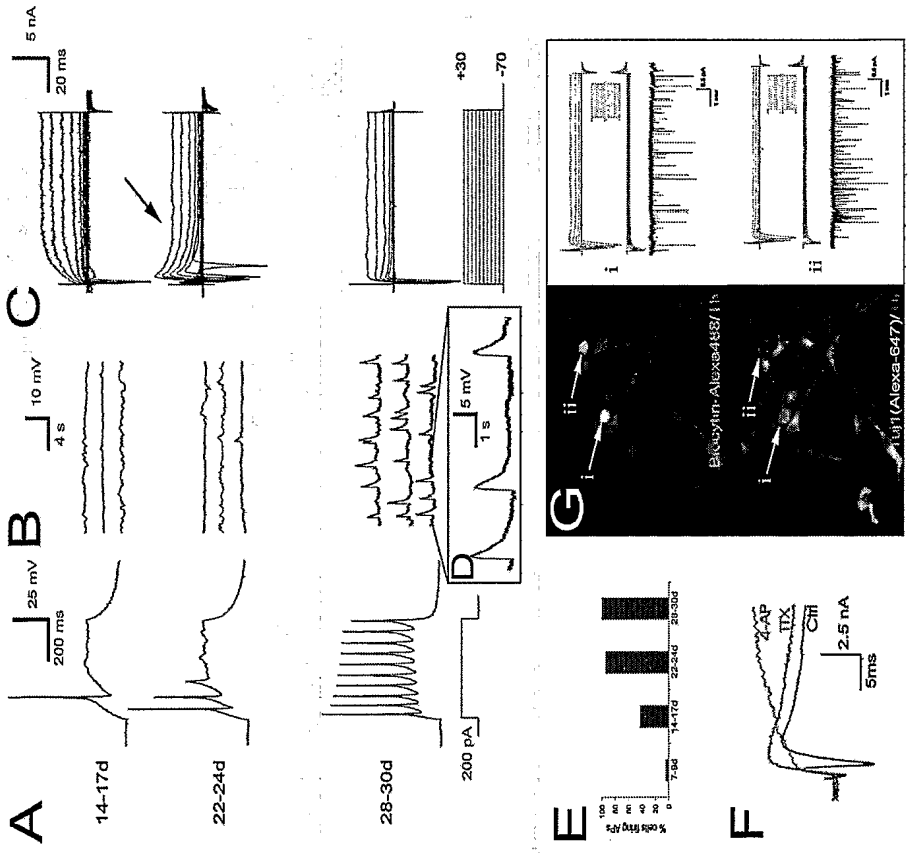

A
B
C
D
E
F
G
25 mV
200 ms
10 mV
4 s
5 nA
20 ms
5 mV
1 s
+30
-70
200 pA
14-17d
22-24d
28-30d
2-AP
TTX
Cm
2.5 nA
5ms
i
ii
Biocytin-Alexa488/
Lgi1(Alexa-647)/

Fig. 7

Fig. 4

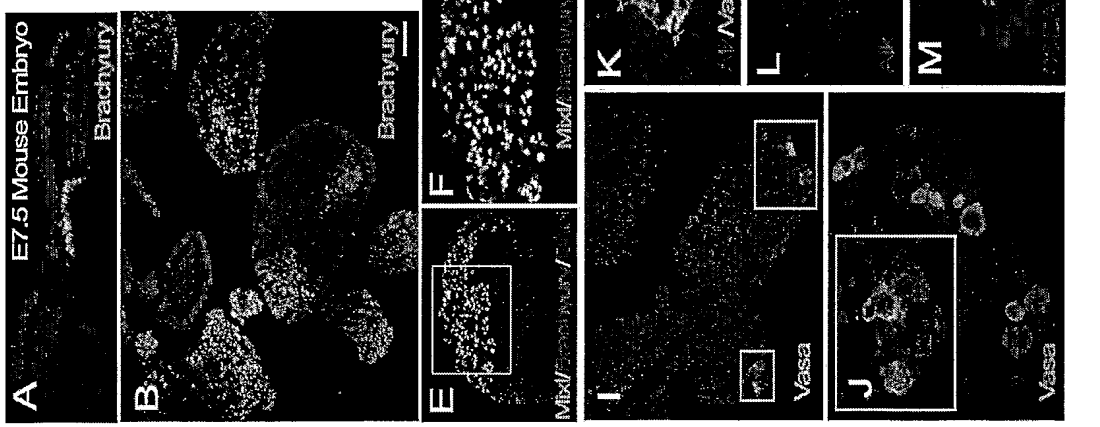

A
E7.5 Mouse Embryo
Brachyury
B
Brachyury
C
GATA4+ AFP-
Brachyury
% of Cell in EBs
40
30
20
10
0
D
Control
Serum
Serum
FGF4
Control
Days
2
4
8
E
Mixl/
F
Mixl/
G
/Mixl
H
/Brachyury
I
Vasa
J
Vasa
K
/Nasa
L
M
/Nasa
N
/Nasa
O
% of EBs with VASA
80
60
40
20
0
2/221
0/348
15/367
66/216
170/237
Control
FGF4
Serum
LIC1
BMP4

Fig. 8

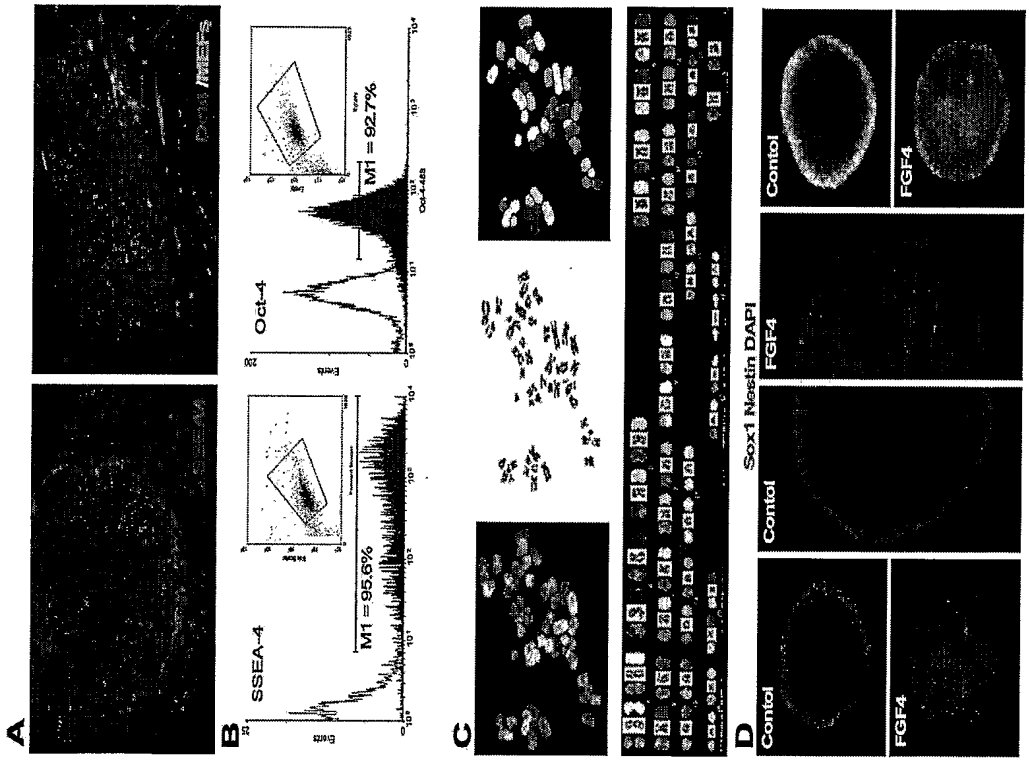
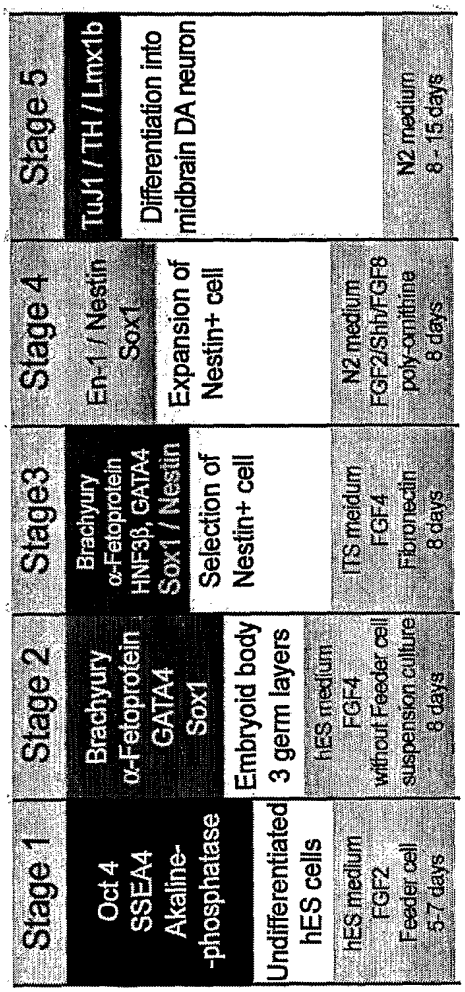

A
B
SSEA-4
M1 = 95.6%
Oct-4
M1 = 92.7%
C
D
Sox1 Nestin DAPI
Contol
FGF4
Contol
FGF4

Fig. 9

Fig. 10



	Control	LiCl	FGF4	BMP4	Serum	Nodal	Nodal+FGF4
Sox1	-		++ ↓				
Brachyury	-	-	+++ ↓	-	+++ ↓		
AFP/GATA4 (O)	-	+++ ↓	-	++ ↓	++ ↓		
GATA4 (I)	-	++ ↓	-	-	+++ ↓		

- No change
+ <50%
++ 50-100%
+++ > 100%

SEQUENCE LISTING

<110> THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES
Kim, Jong-Hoon
Kittappa, Raja
McKay, Ronald D.

<120> METHODS FOR THE DIFFERENTIATION OF HUMAN STEM CELLS

<130> 4239-64833-02

<150> US 60/495,346
<151> 2003-08-14

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